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Does a Progesterone Receptor Mechanism Maintain Pregnancy?

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Honors Thesis

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Abstract

Progesterone is a gonadal steroid that maintains pregnancy and prevents ripening of the cervix. Previous research has shown that progesterone has a strong binding affinity for both progesterone and glucocorticoid receptors. However, what is not known is the role these receptors play in the parturition process. The purpose of this study was to determine whether progesterone withdrawal promotes the neuroinflammatory processes within the pre-partum cervix through the actions of progesterone receptors. To test this, time-dated pregnant mice were subjected to pure agonists or a progestagen that binds to both receptors. Sections of cervix were analyzed to count resident immune cells and determine ripening within each cervix. Immune cell counts did not display any significant differences between the mice treated with the pure progesterone agonist and the mice treated with the mixed progestagen. Cell nuclei counts revealed a significant increase in cell nuclei of the pre-partum cervices treated with the pure progesterone agonist. Serum progesterone levels behaved as expected. Further analysis of cervical tissue will be performed in order to improve the validity of the results found in this study.

Introduction

Roughly 12.4% of the births in the U.S. today are premature births – a number that is strikingly higher than many other developed nations (Grady, 2009). A premature birth is defined as occurring before 37 weeks of gestation. While the reasons for the high premature birth rate in the U.S. are unclear, many believe the largest contributor is the increased number of late pre-term births, which refers to births occurring between 34 and 36 weeks of gestation. Several factors are considered to contribute to late pre-term births including an increase in multiple births, a rise in assisted reproductive technologies, health insurance status, and an increase in women choosing to induce labor (Park *et al.*, 2009). While this last reason may seem unusual, women may choose to induce preterm labor in cases of a uterine infection, amniotic sac rupture, placental deterioration, and when the mother has medical conditions that could harm the baby, such as hypertension (Mayo Clinic, 2010).

Premature labor and birth have both been implicated in fetal morbidity and mortality (McDonald *et al.*, 1996). One reason for this has to do with the development of the lungs while the fetus resides in the uterus. The lungs are the last organs in the mammalian fetus to fully develop. One vital step in lung maturation is the production of surfactant, a lipoprotein responsible for reducing the alveolar surface tension, which is necessary for respiration to occur (West *et al.*, 1994). This and other vital steps in lung maturation must occur before the process of parturition in order for the infant to draw in its first breath. Failure for this to occur results in the death of the newborn due to deficient amounts of oxygen being delivered to the brain and other vital organs (Gilbert, 2006). Lack of maturation prior to birth can also cause life-long illnesses, such as

Respiratory Distress Syndrome, Chronic Lung Disease, and even Cerebral Palsy (Fraser *et al.*, 2004; 4MyChild, 2010). Premature birth can also lead to delayed development and learning disabilities later in life (March of Dimes, 2009).

Current management techniques for preterm labor are aimed at delaying birth once labor has already begun by treating the mother with anti-contraction medications known as tocolytics (Simhan *et al.*, 2007). Types of tocolytics include magnesium sulfate, which aims to stop uterine contractions by blocking neuromuscular transmission, and indomethacin, an agent used to stop the production of cytokines and halt the neuroinflammatory response (Von der Pool, 1998). Other methods attempt to speed up the maturation of the baby's lungs through the administration of glucocorticoids, which are steroids capable of inducing the production of surfactant in the fetal lungs. However, these methods are not consistently effective and can result in side effects such as neuromotor and cognitive defects in the child (Yeh *et al.*, 2004). Since little is known regarding the physiological processes within the body that are responsible for causing preterm birth in humans, effective treatments to prevent, rather than just delay, preterm labor and birth have not been developed.

During gestation, the rigid, collagen-rich cervix acts as a gatekeeper between the fetus and the outside world (Yellon *et al.*, 2009). 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 (Leppert *et al.*, 1995). Failure of the cervix to ripen and remodel can result in

dystocia, or difficult labor, and make vaginal births very painful or impossible for the mother (Cunningham *et al.*, 2009). During the time that the cervix is preparing to open, the uterus simultaneously begins to experience increased contractility. These muscle contractions are essential for pushing the fetus through the cervical opening (Mackler *et al.*, 1999).

Progesterone is a female gonadal hormone that plays a vital role in maintenance of pregnancy and prevention of preterm labor. One way that it does this is by inhibiting ripening of the cervix (Chwalisz *et al.*, 1991). In fact, research suggests that progesterone alone is capable of maintaining pregnancy without the aid of estrogen (Luque *et al.*, 1996). Studies have shown that removing the source of progesterone induces parturition in animals such as rabbits and mice. In these studies, this removal usually comes in the form of removing the ovaries. However, the source of progesterone in humans is not actually ever removed when the process of parturition begins. In fact, the plasma levels of progesterone in women who are giving birth are maintained at high levels until the placenta is expelled, suggesting an apparent lack of actual withdrawal of progesterone in humans. This has led to the formulation of the idea of "functional progesterone withdrawal" in humans. This idea simply states that although systemic progesterone levels remain high in the body, the uterus and cervix gradually lose the capability to respond to progesterone's effects, and as a result cervical ripening occurs (Zakar *et al.*, 2007). The causes underlying functional progesterone withdrawal are unknown, but several theories exist including catabolism of progesterone in the uterus into inactive compounds, alterations in the progesterone receptor isoform ratios, and changes in cofactor protein levels affecting progesterone receptor activation (Brown *et al,* 2005). To

mimic the idea of human functional progesterone withdrawal in mice studies, removal of the progesterone source through ovariectomies must occur. Therefore, the term progesterone withdrawal throughout the paper will refer to both systemic progesterone withdrawal, as well as functional progesterone withdrawal, depending on the species in question.

Upon the withdrawal of progesterone, cervical ripening takes place, resembling an inflammatory process in which immune cells, specifically macrophages and neutrophils, migrate into the cervix. (Yellon *et al.*, 2009). Increased activity of macrophages has been correlated with the breakdown of collagen in the cervix. 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 (Busiek *et al.*, 1995). When macrophages become activated, they produce these pro-inflammatory cytokines, such as interleukin-6 (IL-6) and interleukin-8 (IL-8), both of which are responsible for leukocyte recruitment in the cervix (Madigan *et al.*, 2005). As a result of this cell signaling, the inflammatory response within the cervix is amplified. While other leukocytes, such as neutrophils, are also present in the womb during gestation, macrophages appear to be the most prevalent and abundant type of immune cell found in the uterus and cervix. Enzyme digestion of the pregnant murine, or rodent, uterus provides some evidence for this, showing that macrophages comprise 22% of cells in the pregnant murine uterus as compared to only 10% macrophage composition in a non-pregnant murine uterus (Hunt *et al.*, 1985). In the mouse cervix, the greatest number of macrophages is seen on Day 18, the day directly preceding parturition (Mackler *et al.*, 1999).

However, less research has focused on immune cell counts in the human uterus and cervix. One such study focused on the relationship between cytokine production and cervical ripening in humans, in which they found that cervical ripening correlates with an increase in cytokines within the cervix, specifically IL-6 and IL-8. The study attributed the cytokine production mostly to neutrophils, however no measurement or identification of neutrophils in the cervix was ever used in the study. In addition, macrophages are also capable of secreting IL-6 and IL-8 (Sennstrom *et al.*, 2000). Therefore, the possibility that the increase in cytokines seen in this study was due to an increase of macrophages in the cervix, rather than, or in addition to, neutrophils, cannot be excluded. In addition to the link seen between withdrawal of progesterone and migration of immune cells into the womb, the presence of progesterone has also been seen to inhibit immune cell function in the ovine, or sheep, uterus (Padua *et al.*, 2005). This suggests that not only does progesterone prevent the migration of active immune cells into the uterus and cervix; it also prevents the inflammatory activity of immune cells already residing in these areas. Human studies have also shown the existence of progesterone receptors on macrophages using RT-PCR and immunohistochemistry, supporting the idea that immune cells respond to the presence or absence of progesterone (Khan *et al.*, 2005).

Location and activity of macrophages can be analyzed using several different methods. One such method utilizes interstitial cell adhesion molecule (ICAM-1), which is a protein displayed on the surface of active macrophages. It has been observed that macrophages in the womb of laboring women express ICAM-1 at an increased rate, indicating a correlation between increased activity of macrophages and the process of labor (Ledingham *et al.*, 2001). Macrophages and other leukocytes also display CD

marker proteins on their surfaces that can be indicated through the use of antibodies. (Mackler *et al.*, 2000). For example, BM8 is a macrophage marker protein located in the membrane of the cell (Abcam, 2010). Another method to visualize and identify leukocytes is to track the release of cytokines, such as the aforementioned IL-6 and IL-8, by using substances that can bind to these cytokines and then be visualized. Although these techniques allow researchers to obtain information regarding the leukocyte infiltration and inflammatory processes occurring in the cervix during cervical ripening and parturition, exactly how progesterone affects these neuroinflammatory actions is unknown.

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 altering gene expression, which leads to maintenance of pregnancy (Ashley *et al.*, 2009). Two known isoforms of the nuclear progesterone receptors exist in humans: hPR-A and hPR-B. The hPR-B isoform is 164 amino acids longer than the hPR-A, however their difference in function is not completely clear. Research has supported the idea of hPR-B functioning as a transcriptional activator, while hPR-A acts as a dominant negative repressor of hPR-B in some ways (Giangrande *et al.*, 2000; Smith *et al.*, 2002). Further studies are currently being performed and analyzed in order to better understand the specific functions that each nuclear isoform plays in maintenance of pregnancy.

Recent studies have illustrated the ability of progesterone to produce stimulatory effects that occur too rapidly to be a result of nuclear progesterone receptor action. This suggests the existence of cell membrane progesterone receptors (mPR's) that are also

involved in mediating the effects of progesterone (Ashley *et al.*, 2009). This mPR has been seen in several different animals including the sheep, seatrout, and humans (Dong *et al.*, 2010). It is believed that the rapid response of the membrane progesterone receptors occurs through the non-genomic actions of signal transduction pathways. Recent studies regarding membrane progesterone receptors have verified that progesterone is in fact the ligand for mPR. These studies have also investigated the changing of mPR expression in regards to the estrous cycle stages, showing that the expression of mPR in the ovine corpus luteum and uterus was correlated to the different estrous cycle stages (Ashley *et al.*, 2009). Discovery and analysis of membrane progesterone receptors are relatively new and thus little is known about their role in maintenance of pregnancy.

Additional research has illustrated that the progesterone hormone and other hormones within the progestagens group, such as medroxyprogesterone acetate, possess a strong binding affinity for both progesterone receptors and glucocorticoid receptors (Pasqualini *et al.*, 2007). Progestagens are variants of progesterone (Merriam-Webster Online, 2010). Progesterone is a steroid hormone and as such, contains the characteristic 4-ring structure with specific substituents. Glucocorticoids are also steroid hormones with a similar structure and as a result, progesterone and other progestatens are capable of weakly mimicking the actions of glucocorticoids (Ganguly *et al.,* 1981). Medroxyprogesterone acetate is an artificial chemical that has been shown to forestall inflammation-induced cervical ripening (Yellon *et al.*, 2009). In addition, treatment with the pure progesterone receptor agonist, promegestone (R5020), was successful at maintaining an unripe cervix in pregnant rats (Shi *et al.*, 2000). This means that a compound capable of binding only to progesterone receptors was able to maintain

pregnancy, implying that progesterone receptors specifically play a role in the maintenance of pregnancy. Dexamethasone, a pure corticosteroid capable of binding only to glucocorticoid receptors, was used to treat pregnant rabbits in order to investigate its role in determining gestational length. Researchers found that treatment with dexamethasone in the rabbits resulted in premature delivery (Challis *et al.*, 1975). However, in a small trial study of pregnant women treated with corticosteroids, no premature induction of labor or cervical ripening was seen (Kavanaugh *et al.*, 2006). Thus far, there is no clear evidence explaining the role, if any, of corticosteroids and glucocorticoid receptors in the maintenance of pregnancy and an unripe cervix or cervical ripening and the induction of labor.

Mice share many of the same complex physiological systems that humans possess, such as reproductive, immune, endocrine, and nervous systems. In addition to these systems, mice share similarities with the human uterus such as containing different isoforms of progesterone receptors. For these reasons, the mouse has enough commonalities with human physiology to serve as a practical model for this study. In addition, mouse species tend to have a very predictable gestational period, which is generally around 19-21 days depending on the strain of the mouse (NHGRI, 2002). This enables researchers to predict outcomes regarding pregnancy and, specifically for this research project, induce artificial preterm labor. The CD-1 mouse strain that was used in this study has a normal gestational period of 19 days. One reason this strain was particularly useful for this specific study is because this strain had produced predictable results in previous studies regarding maintenance of pregnancy and parturition day using promegestone (R5020) (Philibert *et al.*, 1999). R5020 is a pure progesterone receptor

agonist that was used as a progesterone replacement in this study. The CD-1 strain was chosen for this project because previous studies defined the mode and doses for both the progesterone and estradiol concentrations to sustain pregnancy following ovariectomy.

This specific study explored if the progesterone receptor was responsible for the maintenance of pregnancy, sustains an unripe cervix, inhibits immune cell migration. It is likely that when the responsible receptor is bound, it causes physiological reactions, such as suppression of macrophage activity in the cervix, to take place that prevent the processes leading up to parturition. However, it may be possible that the bound receptor, whether it be the progesterone receptor or the glucocorticoid receptor, is responsible for activation of macrophages and other immune cells, resulting in the inflammatory processes that lead to cervical ripening once progesterone withdrawal occurs. These findings would suggest that progesterone acts as an inhibitor to the receptor, and when the receptor is unbound, it may initiate cervical ripening. The overall goal of this project was to investigate the role of progesterone receptors and glucocorticoid receptors in the determination of gestational length.

Progesterone treatment has been shown to maintain pregnancy by itself and as a result, it is likely that presence of progesterone mediates its effects through the progesterone receptor. In turn, it is also likely that the withdrawal of progesterone mediates the effects of cervical ripening and parturition through the progesterone receptor as well. However, because of progesterone's ability to bind glucocorticoid receptors tightly, the option that glucocorticoid receptors may play a role in maintenance of pregnancy must also be explored. Therefore, the hypothesis tested in this experiment was

that progesterone receptors exclusively mediate the neuroinflammatory processes that result in remodeling of the cervix and 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. Surgery was performed on day 16 of gestation to eliminate the source of natural progesterone and estrogen. Mice were anesthesized with isofluorine, a portion of the back shaved below the last rib, and skin cleaned with ethanol and betadine. Ovariectomies were then performed by making a small incision in the skin of the mouse's back and making a second small incision in the interperitoneal cavity wall directly below the first incision. The ovary appears as a small, follicle-filled pink sphere in a white fat mass and was easily visible through the peritoneal wall and after incision. To remove the ovary, the uterine tube was ligated, then cut just below the ovary using surgical scissors. This procedure was repeated on the opposite side. The peritoneal cavity wall was then sutured. Silastic capsule implants were inserted subcutaneously, after which the skin was stapled back together. These surgeries have no apparent effect on the offspring viability (Yellon *et al.*, 2009).

There were five different groups of mice distinguished by the treatment they received following the bilateral ovariectomy. The first group was a Sham group that did not actually have their ovaries removed, but were subjected to the same physical stress of the surgery including preparation, incisions, peanut oil-filled vehicle implants, stitches, and staples. The Sham group served as the control group for normal cervical remodeling and time of birth. Pups in this group were expected to present on the morning of day 19.

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The second group was an Ovx/V control group, which consisted of mice who had their ovaries removed and were then treated with a vehicle, which consisted of a capsule filled with peanut oil. This group was intended to mimic a pre-term labor and birth group. The third group was the Ovx/E±P control group. These mice had two estrogen capsules and three progesterone capsules implanted following the ovariectomy. Description of the capsules is presented below. On day 18 of gestation, the three progesterone capsules were removed, which initiated the parturition process. The fourth group was treated with an injection of the progesterone agonist R5020 (Ovx/R5020) at a concentration of 12 mg/kg/day (Philibert *et al.*, 1999). It should be noted that R5020 binds strongly to the nuclear progesterone receptor, but not to the membrane progesterone receptor (Dong *et al.*, 2010). The fifth group was injected with a mixed progesterone/glucocorticoid receptor agonist known as medroxyprogesterone acetate. This group was referred to as Ovx/MPA and received treatment at a concentration of 10 mg/kg/day (Elovitz *et al.*, 2004). Progesterone capsules in the Ovx/E±P group were removed on day 18 (Table 1) to reduce systemic progesterone concentrations and induce parturition.

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

Group	Day 16	Day 16.5	Day 17	Day 18	Day 18.5	Day 19
Sham Control	Surgery	X (PreP)	X (PreP)	X (PreP)		X (PP)
Ovx/V Control	Surgery	X (PreP)	X (PP)			
Ovx/E±P Control	Surgery	X (PreP)		X; -P (PreP)	X (PreP)	X (PP)
Ovx/R5020	Surgery	X (PreP)		X (PreP)	X (PreP)	X (PP)
Ovx/MPA	Surgery	X (PreP)		X (PreP)		X (PP)

Ovx = day of ovariectomy, + = treat, - = withdraw, E = estradiol, P = Progesterone,
R5020 = promegestone, MPA= medroxyprogesterone acetate,
PP = post partum,   PreP = pre partum,   X = harvesting of the cervix

Capsule Preparation

Silastic tubing (1.57mm inside diameter x 3.18 mm outside diameter –
manufactured by Corning) and polyethelene tubing (1.57 mm inside diameter x 2.08 mm
outside diameter – manufactured by Clay Adams) were cut in order to construct steroid-
filled silastic capsules. One end of the tube was heat-sealed using the tip of a hot glue
gun. Vehicle capsules were 1 cm long and injected with plain peanut oil, which was the
solvent used to dissolve the other substances used. Progesterone was dissolved in peanut
oil in a concentration of 1g/mL and injected into the unsealed end of a 1 cm silastic
capsule. Estradiol was dissolved in peanut oil to a concentration of 10 µL/mL and
injected into the unsealed end of a 2 cm silastic capsule. Capsules were then heat-sealed
at the end of injection and washed in 90% ethanol in order to sterilize. A 24-hour
incubation period in phosphate buffer saline (PBS) prior to implantation was performed
in order to create a salt balance and pH balance that allowed successful implantation of
the capsule. The beginning of this study was dedicated to determining the types and
amount of capsules that produced serum concentrations capable of maintaining
pregnancy in the mice. Research has previously reported that these capsules are capable
of sustaining pregnancy in mice (Sharma et al., 1983). However, the actual serum
concentrations of progesterone and estrogen during late pregnancy in mice were
unknown and were measured in this study by drawing blood from the heart before the
cervix was excised.

Tissue Collection and Processing

Following ovariectomies and implant treatments, mice were euthanized using CO_2
gas on days 16.5, 17, 18, 18.5, or 19 as seen in the table above. In the Ovx/E±P control,

progesterone was removed from animals that had not yet been sacrificed in order to induce the process of cervical ripening. The abdomens of the mice were cut so as to access both the abdominal cavity and the thoracic cavity. Blood was drawn from the heart and serum frozen for later assay of progesterone and cortical concentrations. The cervix, attached to roughly 1mm of the lower portions of the left and right uterine horns, was excised and post-fixed in 4% paraformaldehyde. The preserved tissue was sent to a histotechnician in the Loma Linda Department of Anatomy in order to embed the tissue in paraffin wax blocks. This allowed for easier sectioning of the tissue. Once embedded, the tissue was sectioned in 10 µm frontal sections and mounted on slides.

Immunohistochemical Staining

The prepared slides were stained for collagen content using picrosirius red, stained for cell nuclei counts using a hematoxylin counterstain, or processed by immunohistochemistry for macrophages and neutrophiils. The process of staining for macrophages will be explained in detail since macrophage counts will provide the main data used to determine cervical ripening trends. Deparaffinization was performed by baking the slides at 60°C for 30-60 minutes. This was followed by two 10-minute incubations using xylene, two 3-minute washes in 100% ethanol, and two 3-minute washes in 90-95% ethanol. Next, the slides were incubated for two 3-minute washes in 70-75% ethanol and finished with one 30-second rinse in deionized water. A pap pen was then used to create a water resistant boundary around the tissue sections, followed by incubation with proteinase K for 2-minutes. Then 3% hydrogen peroxide was added for 15 minutes followed by three 5-minute washes with PBS. The slides were then incubated with BM8 primary antibody in a 1:800 concentration for 90 minutes and rinsed with PBS

for three 5-minute periods. Next, the slides were incubated with biotyinylated anti-rat secondary antibody in a 1:100 concentration for 30 minutes. Three additional 5-minute PBS washes were performed. Compounds known as ABC and DAB substrate were added with PBS washes in between. Hematoxylin staining occurred for 2 seconds. The slides were then rinsed in running water for 15 minutes. Lastly, the tissue was dehydrated by two 5-minute rinses with 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 had been stained, pictures were taken of the tissues using the Zeiss microscope and the Spot Advanced program. For the macrophage and neutrophil stained tissues, a total of 24 pictures of each animal cervix were taken. Three different tissue sections from the same animal were chosen and eight non-overlapping pictures were taken within each tissue section in order to obtain pictures that were representative of the entire cervix.

Pictures were then uploaded and analyzed on the Image Pro Plus 6.0 program. Collagen content was counted manually. However, macrophage counts and neutrophil counts were automatically counted using the program based on color, shape, and size. Counts were then compiled into an Excel sheet and analyzed using the Graph Pad Prism statistical computer program. A one-way ANOVA and an unpaired t-test were used to determine whether there statistically significant differences existed between the six groups.

Results
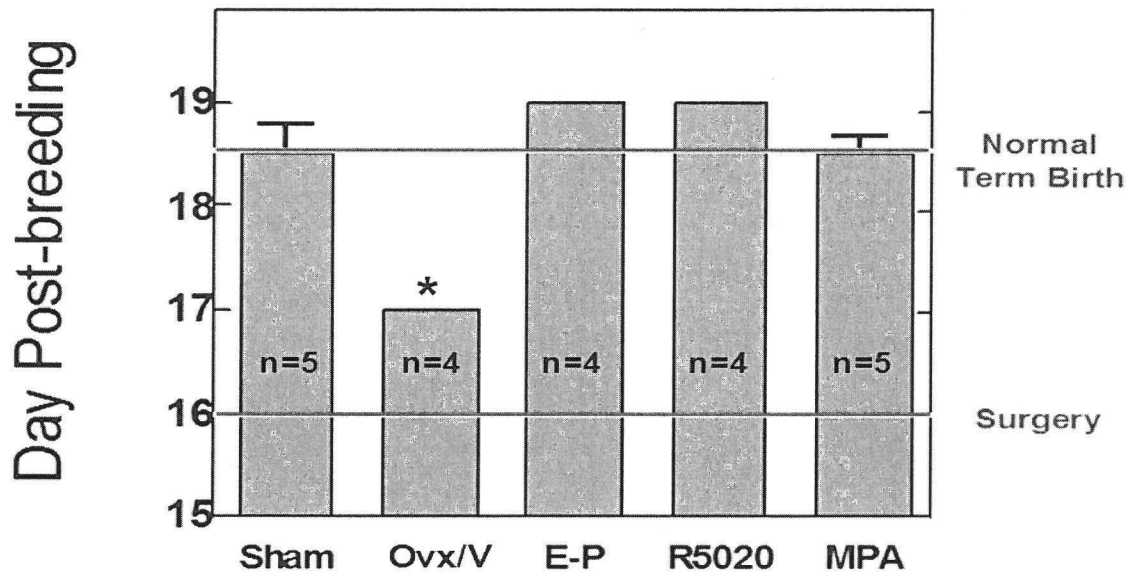
Figure 1. Parturition day of mice subjected to a sham surgery, an ovariectomy (Ovx), an ovariectomy followed by estrogen and progesterone treatment, an ovariectomy followed by R5020 treatment, an ovariectomy followed by MPA treatment, or an ovariectomy followed by dexamethasone treatment. Each bar represents the mean day of birth (±SE, number of mice/group specified). Where SE not shown, all births occurred on the day indicated. * indicates $p < 0.05$ versus sham control. Date of surgery indicated by red line, while normal term birth in blue line is based upon previous reports (Mackler *et al.*, 1999).

Mice in the Sham control group delivered on both Day 18 and Day 19. Expected results based on the hypothesis were seen in the Ovx control group, which delivered on Day 17, as well as the Ovx/E-P3 group, which delivered on Day 19 similar, but not identical, to the sham control. The Ovx/R5020 group experienced parturition on day 19 as expected. The Ovx/MPA group delivered on both Day 18 and Day 19, much like the Sham group, and the Ovx/Dex group delivered on day 17 (Figure 1). A one-way ANOVA of the data revealed that both the Ovx/V group and the Dex group were statistically different from the Sham control.

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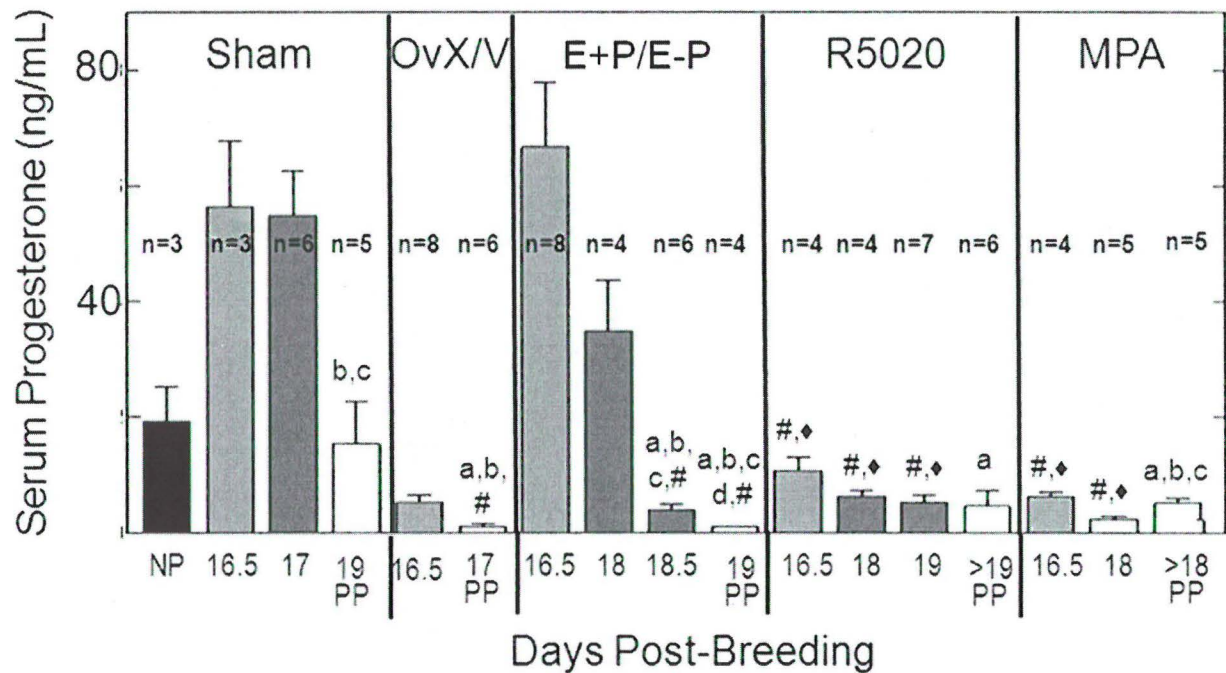
Figure 2. Serum progesterone concentrations of non-pregnant mice or mice subjected to a sham surgery, an ovariectomy (Ovx), an ovariectomy followed by estrogen (E) and progesterone (P) treatment, an ovariectomy followed by R5020 treatment, or an ovariectomy followed by MPA treatment. Each bar represents the mean serum progesterone concentration (±SE, number of mice/group specified). The x-axis indicates the day during gestation that serum levels were measured. a indicates $p<0.05$ versus Non-pregnant. b indicates $p<0.05$ versus day 16.5 within each group. c indicates $p<0.05$ versus day 17/18 within each group. d indicates $p<0.05$ versus day 18.5/19 within each group. # indicates $p<0.05$ versus sham control across groups. ♦ indicates $p<0.05$ versus E+P/E-P across groups. The x-axis indicates the day postbreeding that serum concentrations were measured.

Serum progesterone concentrations displayed expected results (Figure 2). The Sham group shows a gradual decrease in serum progesterone concentrations as gestation progresses. The Ovx/V and MPA groups display low concentrations of serum progesterone immediately following ovariectomies and throughout the rest of the gestational period. The E+P/E-P group shows a progressive decline in progesterone concentrations, much like the sham control, and also illustrates the characteristic drop in progesterone levels on day 18 – the day when macrophage recruitment in the cervix is the highest. The R5020 group displays low concentrations of progesterone immediately

following ovariectomies and throughout the remainder of gestation, much like the Ovx/V

group   (Figure 2).
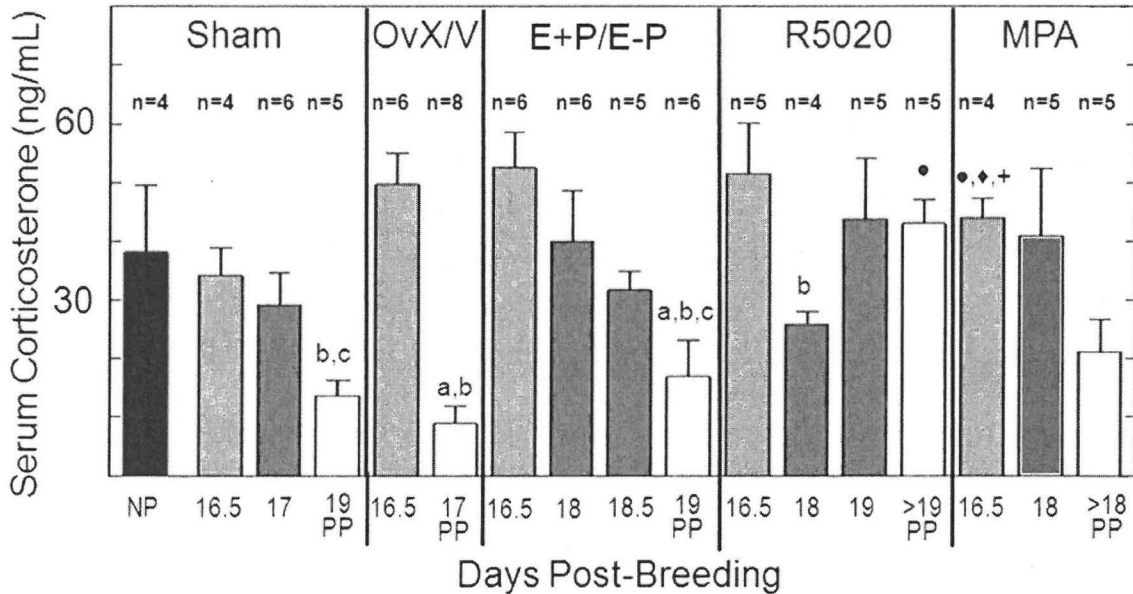
Figure 3. Serum corticosterone concentrations of mice subjected to a sham surgery, an ovariectomy (Ovx), an ovariectomy followed by treatment with estrogen (E) and progesterone (P) treatment, an ovariectomy followed by R5020 treatment, an ovariectomy followed by R5020 treatment, or an ovariectomy followed by MPA treatment. Each bar represents the mean serum corticosterone concentration (±SE, number of mice/group specified). (d#) indicates the day during gestation that serum levels were measured. b indicates $p<0.05$ versus day 16.5 within each group. c indicates $p<0.05$ versus day 17/18 within each group. d indicates $p<0.05$ versus day 18.5/19 within each group. # indicates $p<0.05$ versus sham control. ● indicates $p<0.05$ versus Ovx/V. ◆ indicates $p<0.05$ versus E+P/E-P across groups. + indicates $p<0.05$ versus R5020. The x-axis indicates the day postbreeding that serum concentrations were measured.

Statistical analyses performed on the serum corticosterone concentration data

reveal a decrease in corticosterone concentrations following parturition in the Sham,

Ovx/V, and E-P groups (Figure 3). However, this decrease following parturition is not

seen in the R5020 or MPA groups. The R5020 group actually appears to display an

increase in serum corticosterone concentrations following parturition. Statistical analysis

of the R5020 and MPA groups also fails to show any significant differences between

these groups and the Sham control (Figure 3).
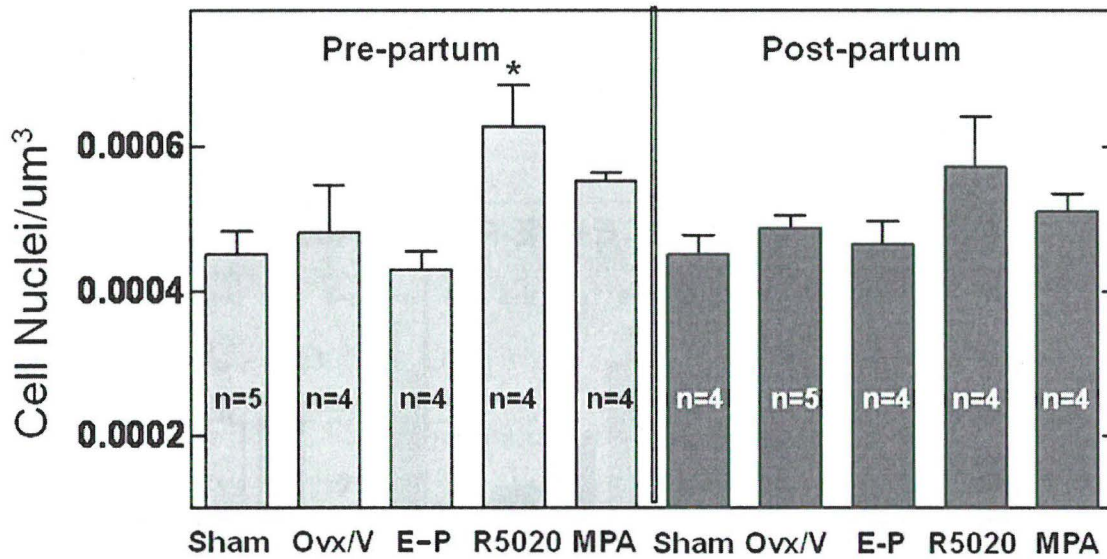
Figure 4. Cell nuclei counts for pre-partum and post-partum groups of mice subjected to a sham treatment, ovariectomy (Ovx), ovariectomy followed by estrogen (E) and progesterone (P) treatment, ovariectomy followed by R5020 treatment, or an ovariectomy followed by MPA treatment. Each bar represents the mean cell nuclei counts per μm^3 of tissue. ($\pm$SE, number of mice/group specified). * indicates $p < 0.03$ between R5020 and E-P. Sham pre-partum refers to gestational day 17, while sham post-partum refers to gestational days 18 and 19. Ovx/V pre-partum refers to gestational day 16.5, while Ovx/V post-partum refers to gestational day 17. E-P pre-partum refers to gestational day 18.5, while E-P post-partum refers to gestational day 19. R5020 pre-partum refers to gestational day 18, while R5020 post-partum refers to gestational day 19. MPA pre-partum refers to gestational day 18, while MPA post-partum refers to gestational day 18.5.

Cell nuclei counts appear to show an increase in cell nuclei numbers in both the R5020 pre-partum and post-partum groups (Figure 4). A one-way ANOVA performed within Pre-partum group indicated a significant difference between the E+P group and the R5020 group (p = 0.0275). However, no other statistical differences were found within the pre-partum group or within the post partum group. A t-test was performed across groups, comparing respective pre-partum and post-partum groups. For example, the pre-partum R5020 was statistically compared to the post-partum R5020 group. No significant differences were found (Figure 4).
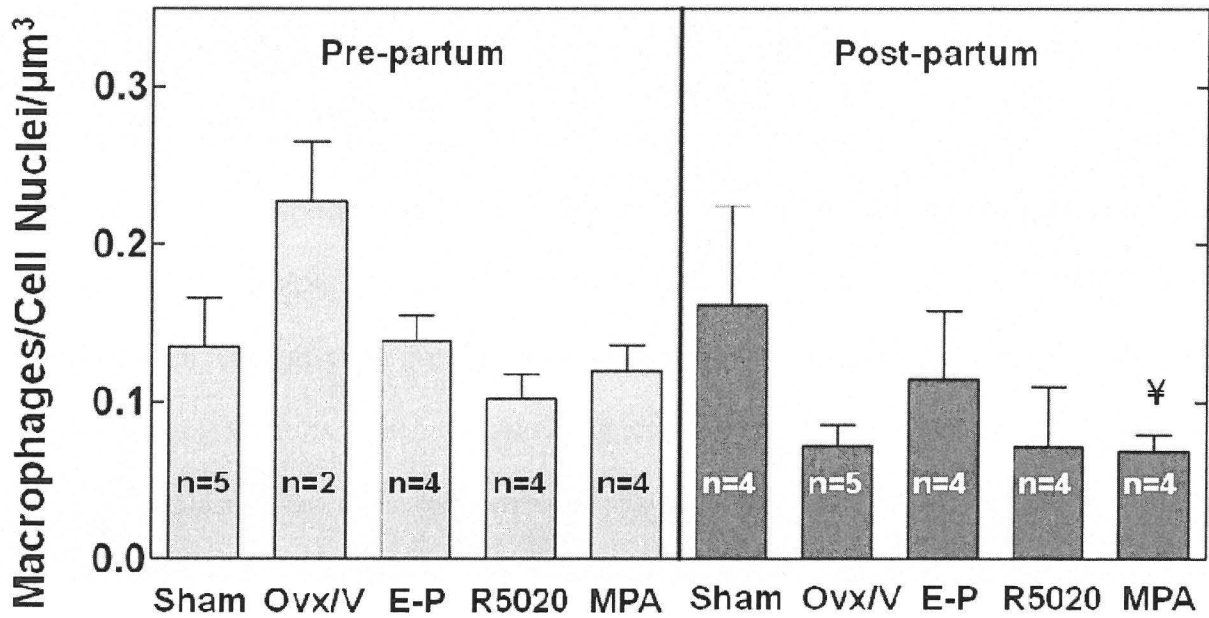
Figure 5. Macrophage counts of pre-partum and post-partum groups of mice subjected to a sham treatment, an ovariectomy (Ovx), and ovariectomy followed by estrogen (E) and progesterone (P) treatment, an ovariectomy followed by R5020 treatment, or an ovariectomy followed by MPA treatment. Each bar represents the mean macrophage counts normalized to cell nuclei counts per μm^3 of tissue (±SE, number of mice/group specified). ¥ indicates $p < 0.04$ between the pre-partum MPA group and the post-partum MPA group. Gestational days for the pre-partum and post-partum groups are the same as in Figure 4.

Macrophage counts display expected trends for the most part (Figure 5). For the Ovx/V, E-P, R5020, and MPA groups, a decreasing trend in normalized macrophage numbers appears to occur from pre-partum to post-partum. However, statistical analysis between the pre-partum and post-partum groups for the Sham, Ovx/V, E-P, and R5020 using an unpaired t-test indicated no statistical differences. On the other hand, an unpaired t-test comparing the pre-partum and post-partum MPA groups revealed a significant decrease in macrophages in the post-partum uterus ($p = 0.0347$). A one-way ANOVA within the pre-partum group showed no significant differences between the groups. Significant differences within the post-partum group were also not seen using a one-way ANOVA (Figure 5).

Discussion

Findings in this study support the hypothesis that progesterone receptors solely mediate the effects of progestational agents to sustain an unripe cervix and forestall cervical ripening. This was shown by the ability of R5020, a chemical that can bind only to progesterone receptors, to maintain pregnancy. Furthermore, R5020 has a strong binding affinity to only the nuclear progesterone receptor, indicating that the nuclear progesterone receptor alone can mediate processes that delay parturition to normal term.

To determine if the progesterone receptor alone was capable of maintaining pregnancy, the observed parturition days of each group were compared (Figure 1). Mice subjected to a Sham surgery gave birth on both days 18 and 19. Mice receiving no treatment were expected to experience normal gestation and parturition to take place on day 19 post-breeding (Philibert *et al.*, 1999). However, delivery on day 18 as well did not prevent conclusions regarding the ability to maintain pregnancy from being made. Because mice in the Sham group did not have their ovaries removed, the observed day of parturition was a result of natural progesterone and natural processes within the body. Based on this information and the statistical analyses performed versus the Sham control, the E+P/E-P, R5020, and MPA groups displayed the ability to maintain pregnancy through term and removal of these substances induced cervical ripening (Figure 1).

Serum progesterone concentration assays revealed low progesterone concentrations for both the R5020 and MPA treatment groups following ovariectomies (Figure 2). This was expected since the natural source of progesterone was removed and

replaced with artificial chemicals that neither contain progesterone nor cross-react with the progesterone assay. These findings indicate that synthetic chemicals unable to sustain circulating progesterone concentrations were still capable of sustaining the progestational effects of progesterone and maintaining pregnancy. Furthermore, both of these chemicals were capable of binding to the progesterone receptor, supporting the idea that the progesterone receptor is responsible for maintenance of pregnancy.

The ability of R5020 to maintain pregnancy indicates that an artificial chemical that binds only to nuclear progesterone receptors was able to maintain pregnancy. This means two things – that a progesterone receptor mechanism alone is responsible for maintaining pregnancy and that this mechanism occurs through the nuclear progesterone receptor and not the membrane progesterone receptor. Therefore, it can be concluded that the effects of the membrane progesterone receptor mentioned earlier are not vital in the processes that maintain pregnancy (Dong *et al.*, 2010). This conclusion allows future research involving preterm birth to be focused on nuclear progesterone receptors and the mechanisms by which they maintain pregnancy.

In order to obtain information regarding the mechanism by which R5020 maintains pregnancy, cell nuclei counts and macrophage counts were performed. R5020 treatment resulted in an increase in cell nuclei numbers, contrary to what is normally observed (Figure 4). The hypertrophy of the cervix as gestation progresses generally results in a decrease in cell nuclei numbers per unit of cervical tissue. However, a significant increase in cell nuclei numbers was observed in mice treated with R5020, suggesting that R5020 maintains pregnancy through a mechanism that increases the number of cell nuclei within the cervix. Whether or not this increase in cell nuclei has

adverse effects on the pregnancy is unknown. Further research must be conducted in order to determine the implications of this increase in cell nuclei numbers.

Macrophage counts for mice treated with R5020 were not statistically significant from the Sham control (Figure 5). This indicates that the number of macrophages recruited within the cervix during a normal mouse pregnancy did not differ significantly from the number of macrophages recruited within the cervix of a mouse treated with R5020. This further suggests that the mechanism by which macrophage recruitment within the cervix occurs in these two treatment groups may be the same or similar. Therefore what is known about the mechanism of R5020 is that it increases the number of cell nuclei present within the cervix and behaves similarly to the Sham control in regards to macrophage recruitment.

The significance of MPA being able to bind to both progesterone and glucocorticoid receptors is that it is possible that MPA exerted its pregnancy maintenance effects through the combined actions of both receptors. Based on this, the next question researchers asked was whether R5020 and MPA acted through the same mechanism to maintain pregnancy. To determine if a difference in mechanism existed, researchers looked at both cell nuclei counts and macrophage counts. As mentioned earlier, R5020 appears to increase cell nuclei numbers within the cervix. However, statistical analysis revealed no difference between the cell nuclei counts of the R5020 and MPA groups, indicating that the pregnancy maintenance mechanism of each compound did not differ with respect to cell nuclei numbers (Figure 4). When comparing macrophage counts of the MPA and R5020 pre-partum groups, no statistical difference was seen between the two, indicating that macrophage recruitment does not differ in cervices treated with these

two chemicals. These results suggest that the mechanisms by which these R5020 and MPA maintain pregnancy do not differ with respect to the cell nuclei numbers or the recruitment of macrophages. However, it is still possible that the mechanisms may differ with respect to collagen breakdown or nerve fibers and further analysis must be done to determine if these two mechanisms truly are the same. If so, researchers could conclude that MPA maintains pregnancy solely through the nuclear progesterone receptor just as R5020 does.

Less in known regarding corticosterone levels throughout gestation. Therefore, expected results were not established prior to the assays. A spike in corticosterone concentrations is seen immediately following surgery in the Ovx/V, Ovx/E±P, and Ovx/R5020 groups (Figure 3). This spike may be a result of the surgery, but this is unlikely since it does not occur in the Ovx/MPA group as well. Pre-partum days 18, 18.5, and 19 are similar for all groups indicating that the specific treatments do not affect the pre-partum corticosterone concentrations. This provides further evidence that the glucocorticoid receptor does not play an integral role in the processes leading up to parturition.

Several animals that were used in this study have yet to be analyzed. As a result, a lack of data prevents solid conclusions from being made at this point, but trends and statistical differences can be seen and explored. In addition some cervices were preserved better than others, meaning that the tissue was easier to section and visualize. In some cases, cervices were not fully intact and as a result, some the images being analyzed had "holes" in the tissue. This was avoided for the most part by using parts of the cervix that

were intact for analysis, but in cases where this was unavoidable, cervices were analyzed as accurately as possible.

Further analysis is necessary to determine if progesterone is acting only through the progesterone receptor alone, or if it is capable of acting through the combined actions of both the progesterone receptor and the glucocorticoid receptor. Collagen and nerve fiber data have yet to be collected and analyzed. These data may provide some insight into possible differences between the mechanisms of R5020 and MPA, as well as provide useful information regarding the mechanisms involved in pregnancy. 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. In addition, it would be useful to determine the exact role, if any, that the membrane progesterone receptors play in the in pregnancy and the specific pathways by which these mPR's work. 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.

In summary, the results of the present study demonstrate that the nuclear progesterone receptor alone is capable of maintaining pregnancy. In addition, these results exhibit the ability of an artificial chemical to maintain pregnancy. This result suggests that treatment with R5020 may have the ability to forestall preterm birth in humans. Determining the mechanism by which R5020 prevents preterm birth will not only be important in understanding of the physiological processes involved in pregnancy,

but has implications in decreasing the rate of preterm births and improving neonatal outcome in the future.

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