PRELIMINARY SAFETY EVALUATION OF 17-HYDROXYPROGESTERONE CAPROATE (17-OHPC) INTRAVAGINAL GEL IN PREGNANT RATS

by

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Preterm birth is defined as delivery before 37 weeks of gestation. The only current FDA approved treatment option for the prevention of preterm birth is a 250 mg weekly intramuscular injection of an oily formulation of 17-hydroxyprogesterone caproate (17-OHPC) sold under the trade name Makena®. This treatment strategy requires the subjects to make weekly hospital visits and in addition, pain at injection site is a common complaint. 17-OHPC has been shown to be effective only in 33% of women receiving the treatment. Recently, the FDA approved 17-OHPC in a subcutaneous auto injector device (Makena auto-injector). The auto-injector device offers some potential advantages, but patients still need to visit the hospital to receive the injection. The injection site pain continues to be a problem with the autoinjector. An alternate route which is safe and effective is needed to help overcome these limitations and benefit a larger proportion of the patient population.

Studies from our laboratory have evaluated the pharmacokinetics of 17-OHPC after different routes of administration in rats. The oral route of administration is not practical due to a low bioavailability of 3%. Vaginal administration is a logical choice and we have developed a gel formulation for 17-OHPC for vaginal administration. In a preliminary unpublished study from our lab, we have shown the feasibility and safety of intravaginal administration of 17-OHPC in female non-pregnant rats and rabbits. The goal of the current study was to evaluate the safety of 17-OHPC intravaginal gel in pregnant rats.

A gel formulation of 17-OHPC was administered intravaginally to adult female pregnant sprague dawley rats starting from day 10 of gestation. The treatment was continued until the day of delivery. After delivery, both rats and pups were euthanized. Blood and vital organs were collected from the mother for the estimation of 17-OHPC concentrations using LC-MS-MS and for histopathology. Pups were fixed in formalin and the histopathology was assessed.

Repeated intravaginal application did not alter body weight gain in the mothers. After vaginal administration, 17-OHPC showed high local tissue levels at the site of application with undetectable levels in plasma in the mothers. There were no gross differences in histopathology between control and treated rats indicating no tissue damage at the site of application in the mothers. The weight of the pups from both the groups showed no statistically significant differences. There was no damage to any of the vital organs in the pups and there were no differences in the number of pups delivered in control or treated mothers. This indicates that the intravaginal administration of 17-OHPC gel appears to be safe in pregnant rats and the pups delivered. Having established the safety, we plan to initiate a safety study in non-pregnant women before conducting a clinical study in pregnant women.

Table of Contents

List of Tables

List of Figures

Preface

I would like to thank the School of Pharmacy, University of Pittsburgh for giving me an opportunity to pursue my graduate studies. I express gratitude to my advisor Dr. Venkat for all his support, guidance and motivation. I am grateful to have been his student. I thank Dr Steve Caritis for all his support and input in this project. I would like to thank Dr Lisa Rohan and her lab members for their assistance during preparation of the vaginal gel. I would like to thank Dr Lora Rigatti for her help in interpreting the histopathology.

I would like to thank Dr. Imam Hussain Shaik for training me to handle animals and to use LC-MS-MS for analysis of samples. I would like to thank Vignesh Vasudevan for all his help and contribution in this project. I would like to thank all other Venkat lab members for extending a helping hand whenever needed. I would like to thank Dr Maggie Folan and Lori Altenbaugh from the School of Pharmacy for all their help throughout this time period.

Last but not the least, I would like to thank my parents and sister for their unconditional love and support. This would not have been possible without their help and support.

1.0 Introduction

1.1 Preterm Birth and its significance

The gestational length of a normal pregnancy is around 40 weeks and the babies born between 37-42 weeks of gestation are termed full term. Preterm birth (PTB) is defined as delivery before 37 weeks of gestation. According to the recent World Health Organization statistics, approximately 15 million babies are born preterm annually [1]. The preterm birth rate in the US peaked in 2006 at 12.8% and reduced to 11.4% in 2013, but still remains very high [2] .

Preterm birth is an issue of major concern since it is a leading cause of neonatal morbidity and mortality in the United States [3]. Children who are born prematurely have higher rates of cerebral palsy, sensory deficits, learning disabilities and respiratory illnesses compared with children born at term. Preterm infants often require admission to intensive care unit and have longer hospital stays compared to their term counterparts resulting in increased healthcare costs [4].Preterm birth is also associated with numerous long-term health impairments and adult-onset diseases such as hypertension, obesity, and diabetes. The cost of preterm birth in the United States is estimated to be $26 billion as of 2005[5].

1.2 Mechanisms of preterm birth

Preterm birth is classified as spontaneous or indicated based on the obstetric precursors leading to delivery. Preterm premature rupture of membranes (PPROM) or preterm labor with

cervical dilation leads to spontaneous preterm birth. PPROM is the spontaneous rupture of membranes at less than 37 weeks of gestation and 1 hour before contractions [6].The reasons for indicated preterm birth include preeclampsia, fetal distress, intrauterine growth retardation, abruptio placentae and fetal death [7] . The precise mechanism of preterm birth is still unknown but there are a number of risk factors associated with preterm birth including general risk factors, obstetric history and pregnancy related factors [8].

General Risk Factors

The maternal risk factors leading to preterm birth are race, stress levels, social and economic background, age, family history of preterm birth [9]. Although the mechanism is unclear, women with a higher BMI have a greater risk of preterm delivery [10]. Maternal medical disorders such as thyroid disease, hypertension can lead to increased rates of preterm delivery [6]. Preterm birth is associated with periodontal disease although the association is not clear. It may be due to the transmission of the mouth bacteria into the vagina. The risk of preterm birth increases as the disease progresses in pregnancy [11]. Smoking can increase spontaneous preterm birth. Cigarette smoke has chemicals such as nicotine and carbon monoxide which are powerful vasoconstrictors and may lead to placental damage and decreased uteroplacental blood flow [6].

Obstetric History

Women with a previous preterm delivery have a 2.5-fold greater risk of subsequent preterm birth. The risk of subsequent preterm birth is inversely related to the gestational age at previous preterm birth [6]. Women with a short cervix have a greater risk for preterm delivery. The risk is inversely proportional to the size of the cervix [8]. Cervical insufficiency is defined as recurrent second-trimester fetal loss due to the inability of the cervix to maintain pregnancy [12] .Cervical

insufficiency due to congenital cervical weakness, surgery or trauma can result in a greater risk of preterm birth [6].

Pregnancy Related Risk Factors

Multi-fetal gestations carry a substantial risk of preterm delivery and are responsible for 15-20% of preterm births. Multiple pregnancy is more likely than singleton pregnancy to be associated with preterm birth [13]. Nearly 60% of the twins are born preterm. Uterine overdistension and PPROM is believed to be the cause for preterm births in multifetal gestation[6] .

Intrauterine infection is a frequent and important mechanism and accounts for 25-40% of the preterm births. Microorganisms causing the infection are recognized by toll-like receptors which in turn elicit the release of proinflammatory cytokines and chemokines which stimulate the production of prostaglandins and some other inflammatory mediators and matrix degrading enzymes. Prostaglandins cause uterine contractility whereas degradation of extracellular matrix in fetal membranes leads to PPROM [6].

Bacterial vaginosis defined as a change in the microbial ecosystem of the vagina is associated with a 1.5 to 3.5-fold increase in the rate of preterm birth [6, 14]. Asymptomatic Bacteriuria which is persistent colonization of bacteria in the urinary tract in the absence of specific symptoms such as bacteriuria, is associated with increased risk of preterm delivery [8, 15]. Fetal fibronectin is a glycoprotein found in the amniotic membranes, decidua and cytotrophoblasts. Higher levels of fibronectin have been associated with increased risk of preterm birth [8, 16].

1.3 Interventions for prevention of preterm birth

Treatment of Asymptomatic Bacteriuria

Several physiological changes during pregnancy such as dilated ureters, enlarged renal pelvis, displacement of bladder, smooth muscle relaxation leading to decreased peristalsis of ureters and increased bladder capacity may facilitate ascent of bacteria from bladder to the kidney. Without treatment, asymptomatic bacteriuria may proceed to pyelonephritis which is responsible for 5% of preterm births [17]. A short course antibiotic treatment regimen might be helpful for this condition [18].

Smoking Cessation

Tobacco use is related to a wide range of adverse pregnancy outcomes such as intrauterine growth restriction, placental abruption, preterm premature rupture of membranes, low birth weight. Tobacco use is linked to 5% - 8% of PTBs. Smoking cessation in pregnancy can reduce the PTB rate by up to 31%. The physicians should provide proper advice and consult for women to quit smoking[12]. Reduction of smoking in early pregnancy reduced the risk of preterm birth to the level of risk observed in non-smokers[19].

Transvaginal ultrasonography of the cervix

Transvaginal ultrasound measurement is useful for predicting preterm birth. It is a diagnostic tool which is used as a predictor of preterm birth. Current recommendations include initial screening at 16 weeks and repeating every 2 weeks until 24 weeks gestation. Normal ultrasound findings may help prevent unnecessary treatment, including inpatient hospital admissions and tocolysis later in pregnancy[12].

Cervical Insufficiency Treatment

The cervix dilates and opens towards the end of pregnancy to facilitate childbirth. Cervical insufficiency may cause opening of the cervix early which may lead to preterm birth [20].

Cervical cerclage is a medical procedure in which the doctor places a stitch around the cervix to keep it closed. The suture can be removed as the full-term data approaches or if any other medical indications arise[20]. Cerclage placement in women with singleton gestations and a short cervix is another evidence-based strategy to reduce recurrent preterm births [12].

Cervical pessary is a flexible silicone device which may be helpful in reducing cervical insufficiency possibly by relieving pressure on the cervix [8].

Tocolytics

Tocolytic drugs are used to prolong pregnancy in women with an acute risk of preterm birth. Tocolytic drugs do not reduce preterm birth, but delay delivery by about 48 hours [21]. Administration of calcium channel blockers and oxytocin receptor antagonists or beta agonists have been shown to prolong delivery but not prevent preterm birth [22-24].

Progesterone Administration

Progesterone supplementation has shown promising results in reducing preterm birth. Progesterone is responsible for maintaining uterine quiescence during pregnancy and labor is associated with decreased levels of progesterone and increased levels of estrogen [25]. Progesterone has been shown to be effective in reducing the rate of preterm birth in women with a short cervix [26, 27]. The clinical trial conducted by Meis *et al* supported the use of 17-OHPC for prevention of preterm birth where a 33% reduction in preterm birth was observed in women

with a history of prior preterm birth taking weekly injections of 17-OHPC [28]. But, 17-OHPC was ineffective in certain conditions like multifetal gestation and short cervix syndrome [29-31].

1.4 17-OHPC and preterm birth

17-alpha hydroxyprogesterone caproate (17-OHPC) is a synthetic progestogen which is synthesized by acetylation of 17-alpha hydroxyprogesterone with caproic acid in presence of toluene sulfonic acid. It is an agonist of the progesterone receptor which is the biological target of progestogens like progesterone [32]. The exact mechanism of action of 17-OHPC has not been characterized. It is a lipophilic drug and is highly protein bound in blood mainly to albumin [5].17-OHPC was originally introduced clinically in 1956 under the trade name Delalutin®. It was used for the treatment of gynecological and obstetrical disorders such as abortion and early pregnancy loss.

CYP3A4 is primarily involved in metabolism of 17-OHPC in adult hepatocytes and the fetal form of CYP3A4 (CYP3A7) is involved in metabolism of 17-OHPC in fetal hepatocytes [33]. The metabolism of 17-OHPC by hepatic microsomes of human and baboons suggested the formation of mono, di and tri hydroxylated derivatives of 17-OHPC. The metabolism of 17-OHPC by placental microsomes of human and baboons led to the formation of mono hydroxylated derivatives of 17-OHPC. The hydroxy groups are added either on the progesterone nucleus or the caproyl moiety [34]. The effect of 17-OHPC on hepatobiliary transporters in adult and female hepatocytes have identified the inhibition of ABCB11 but the inhibition was observed at higher concentrations indicating no adverse effects are likely at therapeutic levels [35].

The FDA approved formulation used for the prevention of preterm birth in women who have had a prior preterm birth is a 250 mg intramuscular injection that is administered once a week [36]. The treatment typically starts from the 16th week of gestation and continues till the 37th week of gestation or delivery, whichever occurs earlier. Recently, a subcutaneous auto-injector has been developed for 17-OHPC administration in the back of the upper arm. It received FDA approval in February 2018 [37].

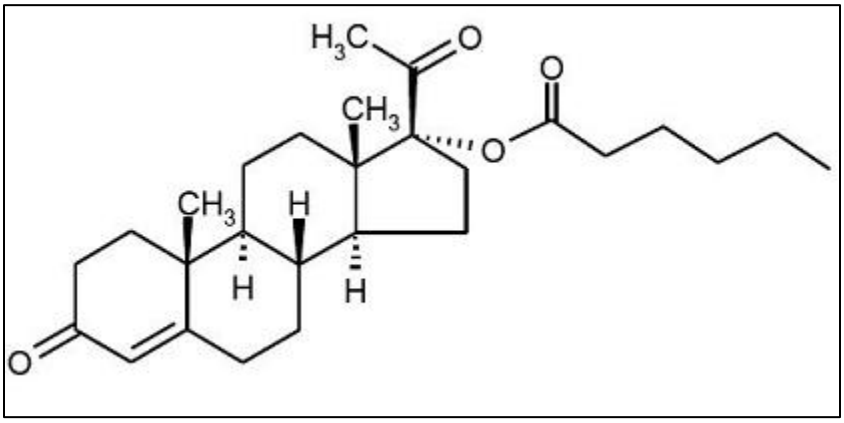

Figure 1: Structure of 17-OHPC [38]

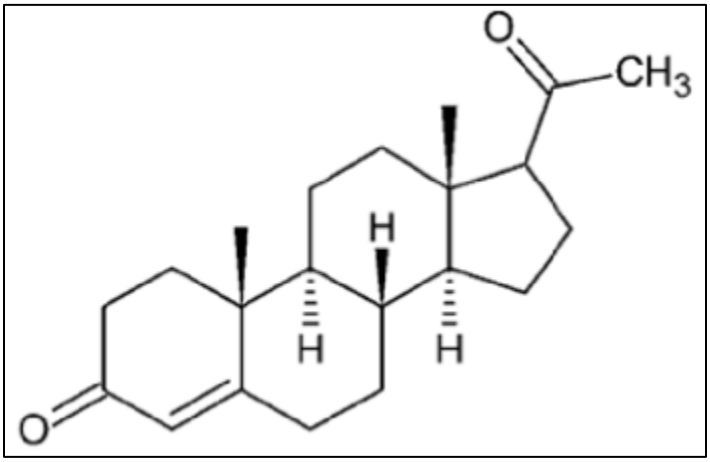

Figure 2: Structure of progesterone[39]

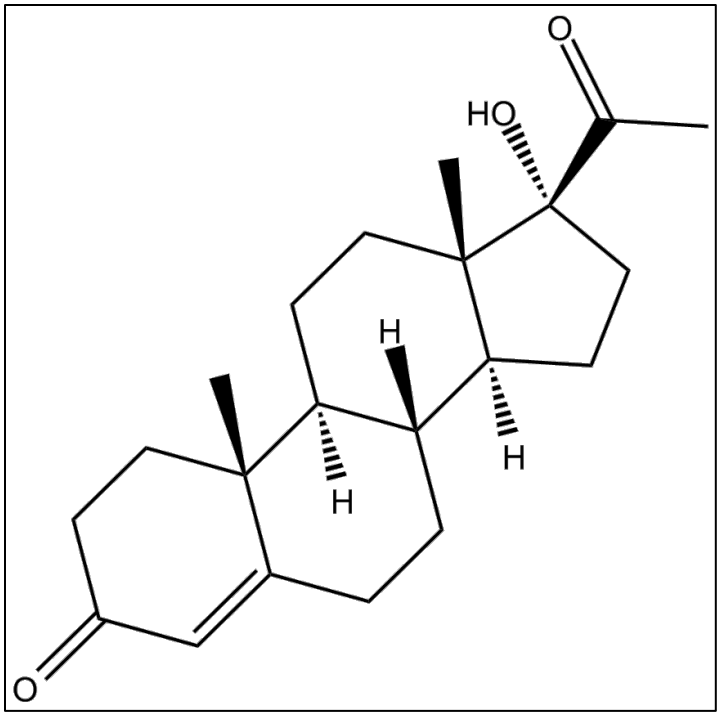

Figure 3: Structure of 17-hydroxyprogesterone [40]

1.4.1 Limitations with existing formulations

Administration of 17-OHPC by intramuscular injections have shown only 33% clinical benefit in pregnant women. There is no clinical evidence to support the superiority of clinical efficiency of the auto-injector over the intramuscular injection. The intramuscular injection has certain drawbacks such as frequent hospital visits, needle pain and large variations in drug exposure as measured by trough plasma concentrations of 17-OHPC [37, 41, 42]. Hence, an alternate route of administration which is safe and effective is needed.

1.4.2 Plasma concentration versus tissue distribution of 17-OHPC after different routes

Studies conducted by Shaik *et al* evaluated the pharmacokinetics of 17-OHPC after various routes of administration. 17-OHPC was administered via the intramuscular, intravenous and oral routes. Intravenous administration lead to an initial distribution phase followed by a slower disposition phase. An initial rapid absorption phase was seen after intramuscular administration of solution dosage form with plasma concentrations declining over time similar to an IV administration. Intramuscular administration of an oil formulation resulted in low and sustained concentrations in plasma over a prolonged time period. Plasma concentrations were undetectable after oral administration of 17-OHPC at 5 mg/kg and the bioavailability was estimated to be only 3% after a dose of 25 mg/kg [36]. These observations ruled out oral route for chronic administration of 17-OHPC. Logically the next route explored was the vaginal route offering the primary advantage of ease of administration. The various vaginal drug delivery systems include suppositories, tablets, creams, gels, films and rings. Crinone® (progesterone), Metrogel® (metronidazole) and EstroGel® (estrogen) are some examples of marketed vaginal gels.

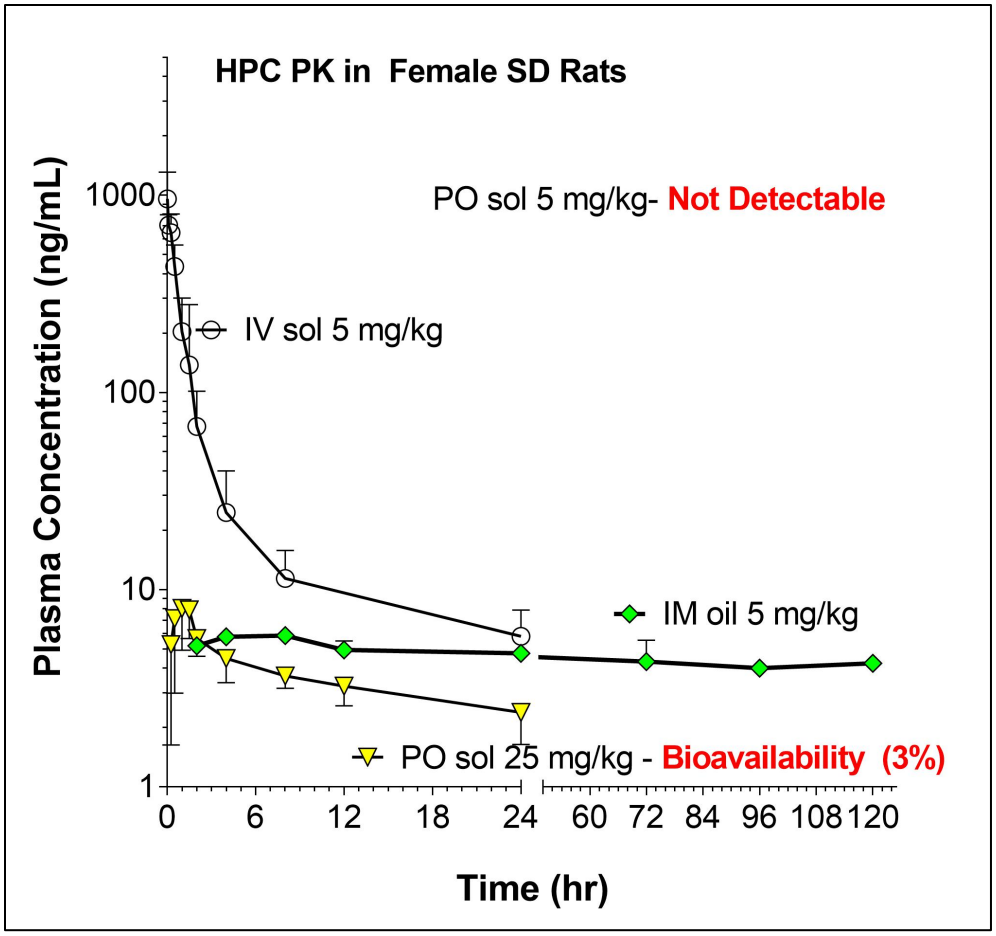

Figure 4:Plasma concentration versus time profile of 17-OHPC after different routes of administration [36]

1.4.3 17-OHPC Vaginal Formulation

The vaginal formulation developed for 17-OHPC should have the following desired characteristics-

- Proper viscosity to avoid leakage from vagina
- Proper bio-adhesive/mucoadhesive properties to increase contact time with vaginal tissue
- Non-toxic and non-irritating
- Compatible with vaginal microflora

- Less frequent administration
- No disruption of innate protective factors
- Ease of administration
- High local exposure
- Low systemic exposure [43]

1.4.4 Objective of the present study

17-OHPC gel was prepared in hydroxyethyl cellulose (HEC), replens and mucolox gel. Preliminary studies were performed in non-pregnant rats and rabbits and the safety of these formulations was established. Studies in non-pregnant rats showed highest levels of 17-OHPC in female reproductive organs after application of mucolox gel. An 8% progesterone gel is used in the clinic for prevention of preterm birth in women with a short cervix. We anticipate that creating a high local exposure in female reproductive organs with 17-OHPC like progesterone may lead to a greater benefit and hence decided to conduct further studies with 8% mucolox gel. The aim of the current study was to ensure safety in pregnant rats since it will be administered to pregnant women.

2.0 Materials and Methods

2.1 Materials

2.1.1 Chemicals

17-OHPC and Mucolox base was purchased from Professional Compounding Centers of America (PCCA), Houston, TX. Formic acid, medroxyprogesterone acetate, ammonium acetate and LC-MS/MS grade solvents of highest purity were purchased from Sigma Chemicals, St Louis, MO.

2.1.2 Animals

Eight adult pregnant female Sprague-Dawley (SD) rats were obtained from Charles River Laboratories, Inc (Wilmington, MA). All the procedures were performed in accordance with the protocol approved by the University of Pittsburgh's Institutional Animal Care and Use Committee (IACUC) and were according to the Guide for the Care and Use of Laboratory animals (National Research Council, 1996, Washington, District of Columbia). The rats were housed in the animal facility and followed a 12 h light/ dark cycle. The rats were allowed free access to food and water ad libitum.

2.2 Methods

2.2.1 Preparation of Vaginal Gel

Table 1 : Formula for 17-OHPC vaginal gel formulation

17-OHPC 8% Gel (Mucolox™/Versabase®)	
Ingredient	**Quantity (For 100 g)**
17-OHPC	8 g
Base, PCCA Mucolox ™	51.1 g
Base, PCCA Versabase® Gel	49.9 g

Mucolox base from PCCA is a base with a unique polymer network that combines the benefits of water solubility and enhanced mucoadhesion properties. Mucolox base is a viscous liquid [44]. The Versabase gel is a clear transparent gel that is added to the mucolox base to form the mucolox gel and also to enhance the mucoadhesion properties. 17-OHPC, PCCA mucolox base and PCCA versabase gel base were added to a mortar and mixed with a pestle to form a white opaque gel ensuring no lumps of particle were formed. The gel was mixed using a spatula before packaging. The gel was filled and sealed with parafilm in 1 ml syringes. The filled syringes were stored at room temperature till use. The blank gel consisted of a transparent gel of PCCA mucolox base and PCCA versabase mixed using a mortar and pestle and filled in 1 ml syringes.

2.2.2 Experimental Groups

The study included a total of 8 pregnant rats which were randomly assigned to two different groups consisting of 4 rats per group. The gestation period in these animals is typically 21-23 days and we wanted to ensure that the animals receive treatment for at least half of the gestational period similar to the current practice in humans. We started treatment in these animals on the 10^{th} day of gestation. The control group of rats was administered only the blank mucolox gel for a period of 10 days or until delivery whichever occurred earlier. The treatment group of rats were administered 8% 17-OHPC in mucolox gel intravaginally for a period of 10 days or until delivery whichever occurred earlier. The mother and pups were sacrificed immediately after delivery. The mother was sacrificed, and blood and tissue samples were collected during the time of euthanasia and stored at -80^0C till the time of analysis. The pups were euthanized by carbon dioxide asphyxiation. The pups were fixed in formalin before sectioning.

2.2.2.1 Observations and Measurements

All animals were observed at least once a day during the study period. Gross maternal body weights were recorded daily. A detailed visceral examination of mothers was conducted. The plasma, uterus, adipose and ovary tissues from mother were analyzed for 17-OHPC concentrations. Litters were assessed for number of implants, resorptions/ dead, normal and abnormal fetuses. A detailed visceral examination of the fetuses was conducted [45].

2.2.3 Sample Analysis

The plasma and tissue concentrations of 17-OHPC were measured using a previously developed and validated LC-MS/MS method in the lab with minor modifications [36, 46]. Partial validation was performed for the assay method used in this study.

2.2.3.1 Standards

The internal standard used was Medroxyprogesterone acetate (MPA). Methanolic stock solutions for primary standards and working solution for internal standard were prepared. The working standards, quality controls and internal standard were stored at -20^{0}C [36].

2.2.3.2 Sample Preparation

Plasma samples were processed using solid phase extraction (SPE) cartridges. One hundred µl of the plasma sample was mixed with 25 µl IS and 1 ml water. Oasis® HLB 1 cc cartridges were washed with 1 ml methanol followed by 1 ml water for preconditioning and then the entire plasma sample mixture was passed through the cartridge. The cartridges were then washed with 1 ml of 95% water and 5% methanol and dried under vacuum. 17-OHPC and IS were collected in a glass tube by eluting with methanol and evaporated to dryness under stream of air. The resultant residue was reconstituted in 100 µl of 50% methanol and 20 µl was injected into the LC-MS-MS system [36].

Fifty mg of adipose or 100 mg of uterus from the cervical end was homogenized in 4 volumes of distilled water respectively using Polytron homogenizer. To 100 µl of tissue homogenate, 25 µl internal standard solution and 500 µl or 300 µl methanol was added to adipose and uterus samples

respectively, vortexed for 30 seconds and centrifuged at 15000 rpm for 5 minutes. Twenty µl of resultant supernatant was injected into the LC-MS-MS system.

Ten mg of ovary was homogenized in 9 volumes of distilled water using Polytron homogenizer. To 100 µl of tissue homogenate, 25 µl internal standard solution and 300 µl methanol were added, vortexed for 30 seconds and centrifuged at 15000 rpm for 5 minutes. Twenty µl of resultant supernatant was injected into the LC-MS-MS system.

2.2.4 Chromatography and mass spectrometry conditions

The chromatographic system used for analysis of 17-OHPC was equipped with a Waters 2795 console (Waters Corporation, Milford, MA). The compounds were separated on a ACQUITY UPLC® BEH shield RP 18 1.7 µm analytical column. The column was maintained at a temperature of 40^0C. The mobile phases were [A] 5% methanol in water containing 2mM ammonium acetate and 0.1% formic acid and [B] 5% water in methanol containing 2mM ammonium acetate and 0.1% formic acid. The sample run time was 10 minutes. The flow rate was 0.3 ml/min with a gradient starting from 30% solution [B] to 95% [B] over 1 min which was held until 4.0 min. The flow returned to the initial condition of 30% [B] in 6.0 min.

Analysis was performed on a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters Corporation, Milford, MA) with positive electrospray ionization mode (+ESI) and multiple reaction monitoring (MRM). The MRM setting used for analyzing the concentrations of 17-OHPC in plasma and tissues were: capillary voltage 3.5kV; source temperature 100^0C; de solvation temperature 450^0C; cone gas flow (l/h) 50; de solvation gas flow (l/h) 550. The LC, mass spectrometer and data collection was performed by Masslynx software version 4.1 (Waters Corporation, Milford, MA) [36].

2.2.5 Statistical Analysis

Results are presented as mean ± standard deviation (SD) or mean ± standard error of mean (SEM) in the figures. Unpaired t-test was used for comparing the means between different groups. Kruskal-Wallis test was used to compare median between two or more groups followed by Dunn's post hoc analysis, with a cutoff of p-value <0.05 considered to be significant. Statistical analysis was conducted using GraphPad Prism 7.

3.0 Results

3.1 Drug administration

The control group received intravaginal administration of mucolox gel for a period of 10 days. The treatment group received 8% 17-OHPC in mucolox gel for a period of 10 days. Two hundred µl of gel was administered to the rats daily. Different applicators were used for administration of the gel to the two different groups of rats.

3.2 Mass spectroscopic analysis

The retention times for 17-OHPC and MPA were 7.4 and 6.2 min, respectively. The mass to charge transitions for these compounds were *m/z* 429.06 → 313.05 for 17-OHPC and *m/z* 387.21 → 327.26 for MPA (IS) [33].

3.2.1 Plasma Assay Validation

Linearity

The ratio of mean peak area of 17-OHPC to the internal standard was linearly related to the concentration of 17-OHPC in the concentration range of 5-1000 ng/ml.

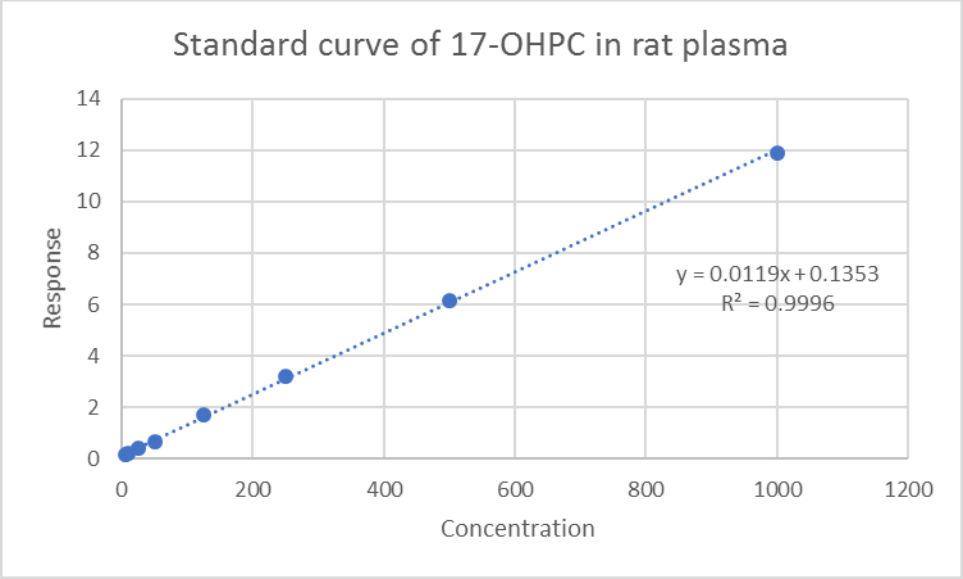

Figure 5:Standard curve of 17-OHPC in rat plasma, showing linearity over a concentration range of 5-1000 ng/ml

Precision and accuracy

Inter-day and intra-day coefficients of variation (CV) were within acceptable limits according to the guidance on bioanalytical method validation which is less than 15% for nominal concentrations and less than 20% for the lower limit of quantification (LLOQ).

Table 2: Intra-day and Inter-day accuracy and precision of 17-OHPC assay in rat plasma

17-OHPC	Added concentration (ng/ml)		
	15	100	900
Intra-day assay			
Mean ± S.D	21.8 ± 0.8	125.7 ± 1.6	978.5 ± 10.8
CV (%)	3.6	1.3	1.1
Inter-day assay			
Mean ± S.D	15.6 ± 0.4	97.2 ± 12.1	883.8 ± 29.2
CV (%)	2.1	12.4	3.3

Recovery

Recovery was determined by comparing the peak areas of plasma samples spiked with 17-OHPC and fixed concentration of internal standard prior to extraction to the non-extracted standard analyte methanolic solutions. The recovery was > 97%.

Ion Suppression

Ion suppression was calculated by dividing the peak areas of blank extracted plasma samples spiked with 17-OHPC and fixed concentration of internal standard after extraction with non-extracted standard analyte methanolic solutions at the same nominal concentration. The ion suppression was <18%.

Table 3: Recovery and ion suppression recovery of 17-OHPC in rat plasma

EXTRACTION RECOVERY (%)		ION SUPPRESSION RECOVERY (%)	
Concentration (ng/ml)	Mean	Concentration (ng/ml)	Mean ± S.D.
15	98	15	>100
45	>100	45	99
90	>100	90	83

3.2.2 Adipose Assay Validation

Linearity

The ratio of mean peak area of 17-OHPC to the internal standard was linearly related to the

concentration of 17-OHPC in concentration range of 5-500 ng/ml.

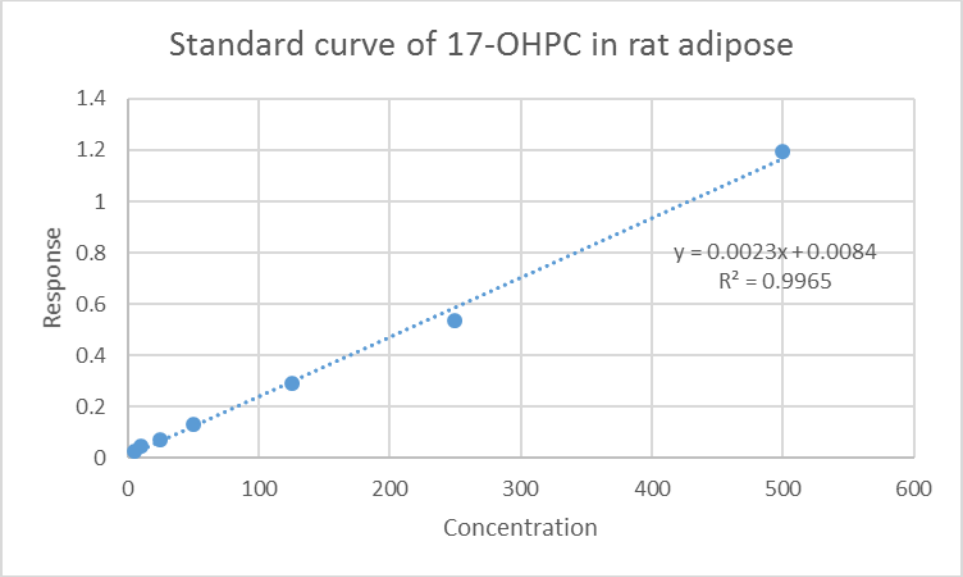

Figure 6: Standard curve of 17-OHPC in rat adipose tissue, showing linearity over a concentration range of 5-500 ng/ml

Precision and accuracy

Inter-day and intra-day coefficients of variation (CV) were within acceptable limits according to the guidance on bioanalytical method validation which is less than 15% for nominal concentrations and less than 20% for the lower limit of quantification (LLOQ).

Table 4: : Intra-day and Inter-day accuracy and precision of 17-OHPC assay in rat adipose tissue

17-OHPC	Added concentration (ng/ml)		
	15	90	450
Intra-day assay			
Mean ± S.D	12.5 ± 1.9	122.3 ± 9.3	523.9 ± 110.8
CV (%)	15.2	7.6	21.1

Inter-day assay			
Mean ± S.D	17.0 ± 1.9	77.1 ± 8.0	439.9 ± 35.9
CV (%)	11	10.5	8.1

Recovery

Recovery was determined by comparing the peak areas of adipose tissue samples spiked with 17-OHPC and fixed concentration of internal standard prior to extraction to the non-extracted standard analyte methanolic solutions. The recovery was > 30%.

Ion Suppression

Ion suppression was calculated by dividing the peak areas of blank extracted adipose tissue spiked with 17-OHPC and fixed concentration of internal standard after extraction with non-extracted standard analyte methanolic solutions at the same nominal concentration. The ion suppression was < 1%

Table 5: Recovery and ion suppression recovery for 17-OHPC in rat adipose tissue

EXTRACTION RECOVERY (%)		ION SUPPRESSION RECOVERY (%)	
Concentration (ng/ml)	Mean	Concentration (ng/ml)	Mean ± S.D.
15	58	15	>100
90	37	90	>100
450	46	450	99

3.2.3 Uterus Assay Validation

Linearity

The ratio of mean peak area of 17-OHPC to the internal standard was linearly related to the concentration of 17-OHPC in concentration range of 5-500 ng/ml.

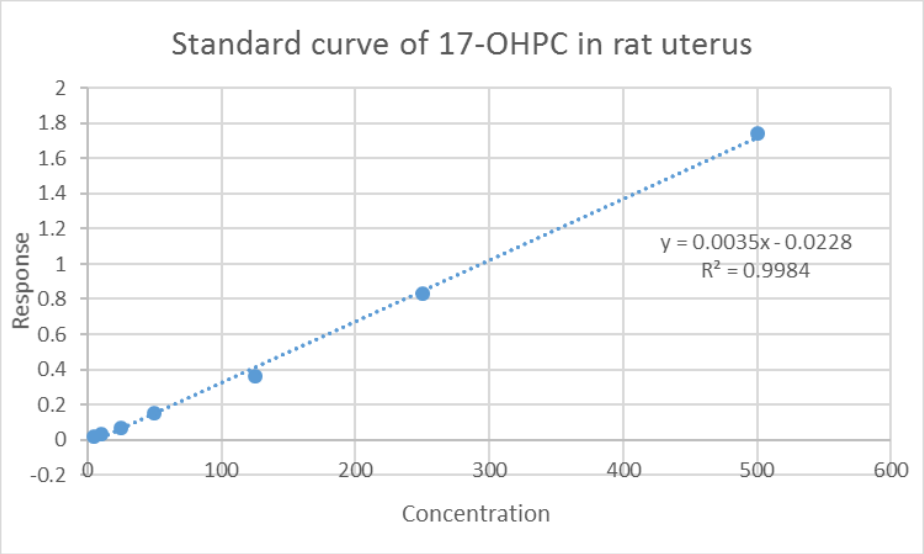

Figure 7:Standard curve of 17-OHPC in rat uterus tissue, showing linearity over a concentration range of 5-500 ng/ml

Precision and accuracy

Inter-day and intra-day coefficients of variation (CV) were within acceptable limits according to the guidance on bioanalytical method validation which is less than 15% for nominal concentrations and less than 20% for the lower limit of quantification (LLOQ).

Table 6: Intra-day and Inter-day accuracy and precision of 17-OHPC assay in rat uterus tissue

17-OHPC	Added concentration (ng/ml)		
	15	90	450
Intra-day assay			
Mean ± S.D	14.7 ± 1.8	85.9 ± 7.9	473 ± 16.7
CV (%)	12.9	9.2	3.5
Inter-day assay			
Mean ± S.D	15.5 ± 2.0	85.7 ± 6.3	449.2 ± 40.7
CV (%)	13.4	7.3	9.0

Recovery

Recovery was determined by comparing the peak areas of uterus tissue samples spiked with 17-OHPC and fixed concentration of internal standard prior to extraction to the non-extracted standard analyte methanolic solutions. The recovery was > 70%.

Ion Suppression

Ion suppression was calculated by dividing the peak areas of blank extracted uterus tissue samples spiked with 17-OHPC and fixed concentration of internal standard after extraction with non-extracted standard analyte methanolic solutions at the same nominal concentration. Ion suppression was <26%.

Table 7: Recovery and ion suppression recovery for 17-OHPC in rat uterus tissue

EXTRACTION RECOVERY (%)		ION SUPPRESSION RECOVERY (%)	
Concentration (ng/ml)	Mean ± S.D.	Concentration (ng/ml)	Mean
15	84	15	74
100	71	100	76
900	82	900	82

3.2.4 Ovary Assay Validation

Linearity

The ratio of mean peak area of 17-OHPC to the internal standard was linearly related to the concentration of 17-OHPC in concentration range of 5-500 ng/ml.

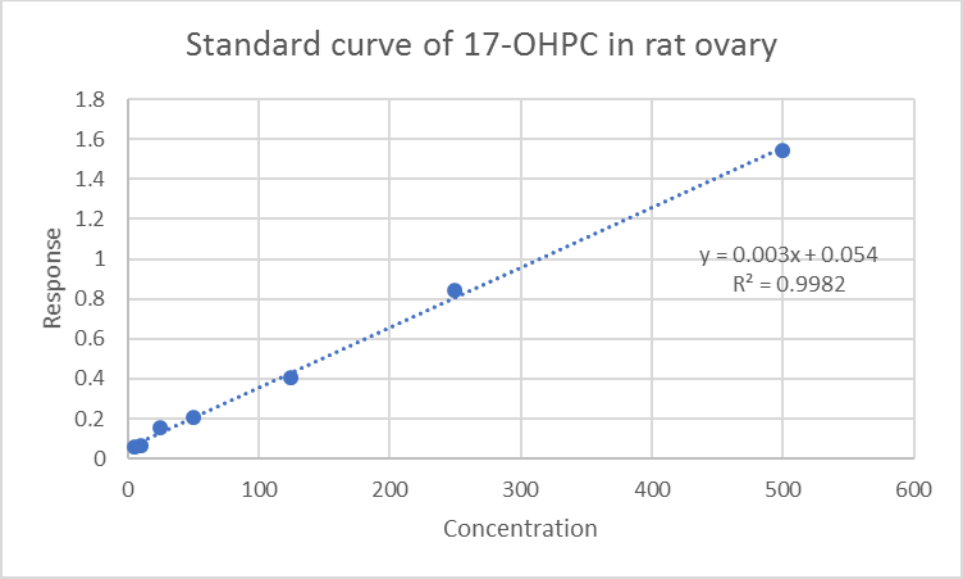

Figure 8:Standard curve of 17-OHPC in rat ovary tissue, showing linearity over a concentration range of 5-500 ng/ml

Precision and accuracy

Inter-day and intra-day coefficients of variation (CV) were within acceptable limits according to the guidance on bioanalytical method validation which is less than 15% for nominal concentrations and less than 20% for the lower limit of quantification (LLOQ).

Table 8: Intra-day and Inter-day accuracy and precision of 17-OHPC assay in rat ovary tissue

17-OHPC	Added concentration (ng/ml)		
	15	90	450
Intra-day assay			
Mean ± S.D	14.9 ± 1.7	86 ± 10.2	432.5 ± 60.7
CV (%)	11.4	11.9	14.0
Inter-day assay			
Mean ± S.D	14.46 ± 1.8	83.63 ± 8.2	464.7 ± 71.9
CV (%)	12.1	9.8	15.4

Recovery

Recovery was determined by comparing the peak areas of ovary tissue samples spiked with 17-OHPC and fixed concentration of internal standard prior to extraction to the non-extracted standard analyte methanolic solutions. The recovery was > 70%.

Ion Suppression

Ion suppression was calculated by dividing the peak areas of uterus tissue samples spiked with 17-OHPC and fixed concentration of internal standard after extraction with non-extracted standard analyte methanolic solutions at the same nominal concentration. There was no ion suppression observed.

Table 9: Recovery and ion suppression recovery for 17-OHPC in rat ovary

EXTRACTION RECOVERY (%)		ION SUPPRESSION RECOVERY (%)	
Concentration (ng/ml)	Mean	Concentration (ng/ml)	Mean
15	99	15	>100
90	89	90	>100
450	73	450	>100

3.3 Maternal Body Weights

Repeated application of 17-OHPC intravaginal gel did not alter the body weight gain in rats. There was no difference in maternal body weights between the control and treated groups.

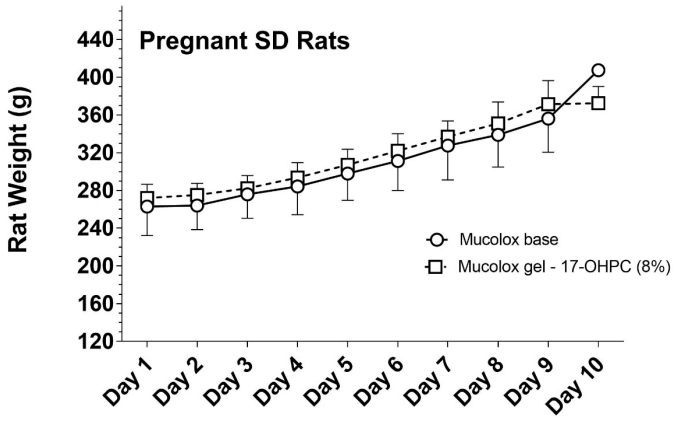

Figure 9:Maternal Body weights of rats receiving vehicle gel or gel with 17-OHPC

3.4 Maternal Plasma and Tissue Sample Analysis

The plasma and tissue samples from the 4 rats in treatment group were analyzed using LC-MS-MS. The plasma levels were below limit of detection of 1 ng/ml. 17-OHPC levels in ovary were below limit of detection of 1 ng/ml. Significant levels of 17-OHPC were seen in the adipose and uterus tissue. The results are presented as mean ± SD.

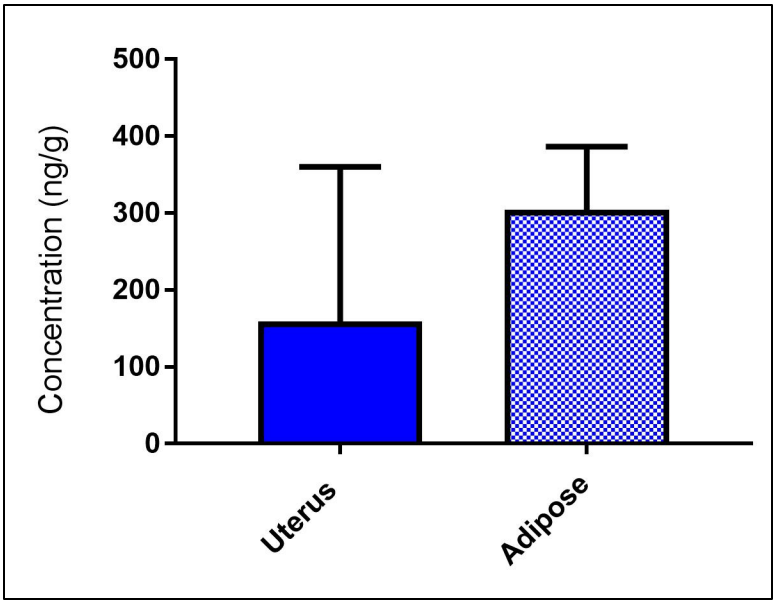

Figure 10:17-OHPC levels in adipose and uterus tissues of mothers receiving 17-OHPC gel

3.5 Maternal Tissue Histopathology

The tissues were scored for epithelial thinning, erosion, exudate, leukocyte infiltration, single-cell necrosis, single-cell vacuolation and luminal blood on a scale of 0 to 4 where 0 stands for no injury and 4 stands for a marked injury. There were no major differences in histopathology scoring between the control and treated groups.

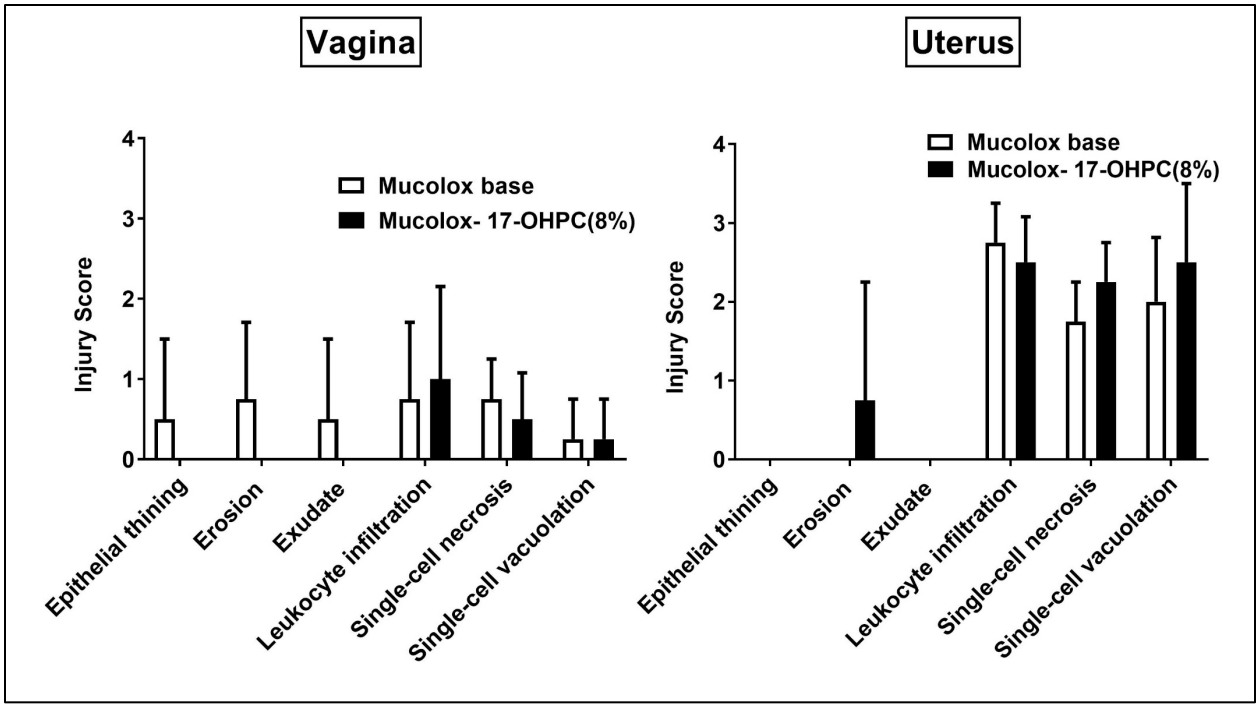

Figure 11:Histopathology of tissues from mother (postpartum) indicating no difference between vehicle gel and gel with 17-OHPC

3.6 Litter Size

The litter size was similar among both the groups with an average litter size of 11 in the control group and 10 in the treated group. An unpaired t-test with Welch's correction was used to check for significance and a p-value of 0.8676 (where $p<0.05$ is significant) was obtained indicating no significance difference between the groups. There was one dead pup in the control group. The results are presented as mean ± SD.

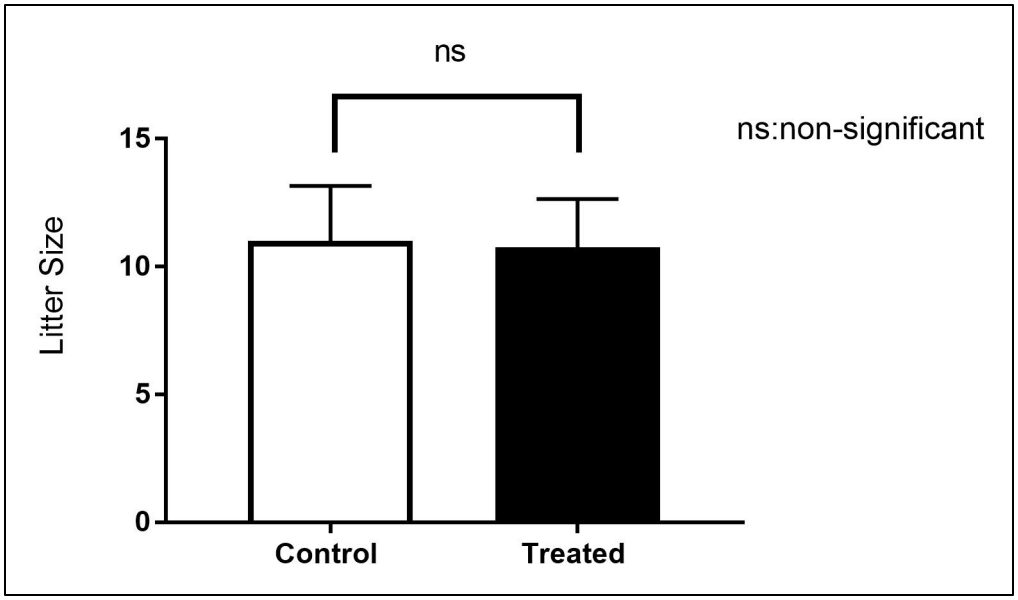

Figure 12:Litter size in control and 17-OHPC treated groups are not different

3.7 Weight of the pups

An unpaired t-test with Welch's correction was used to check for significance where a p-value <0.05 was considered to be significant. The mean weight of the litter from the control group was 3.85 and that of the treated group was 3.62. The weight of the litters from the control and treated groups was not significantly different with a p-value of 0.0708 . The results are presented as mean ± SD.

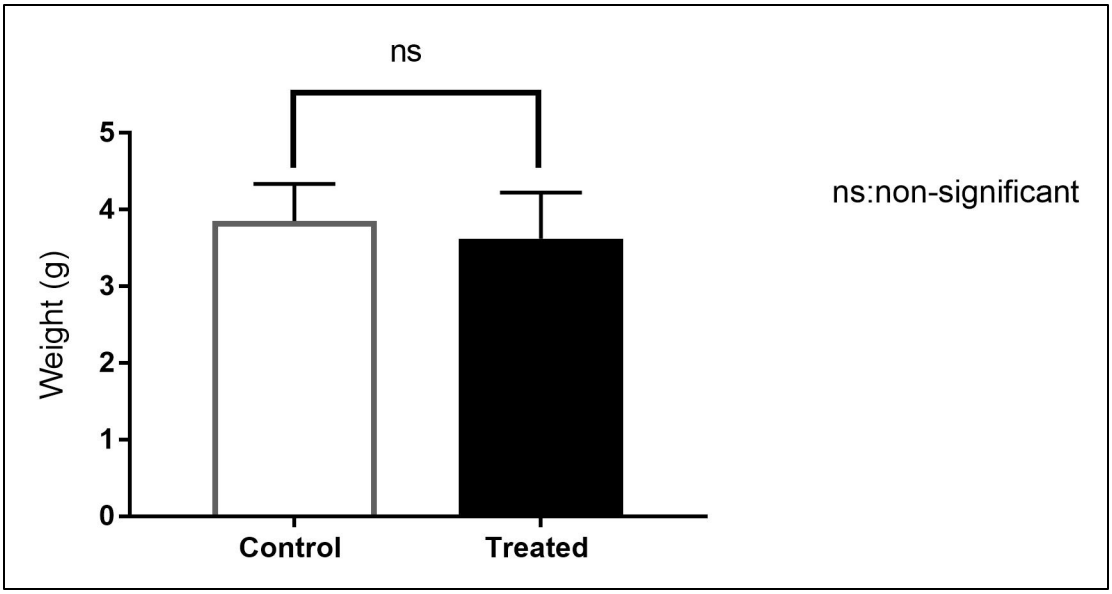

Figure 13:Weight of pups from both control and 17-OHPC treated group are not different

3.8 Histology of pups

The brain, lungs, heart, liver and gonads from the fetuses were examined for visceral malformations and scored on a scale of 0 to 4. Zero stands for no injury and 4 stands for a marked injury. No significant findings were seen indicating no injury.

Table 10: Histopathology of vital organs of pups

	Control				Treated			
	Dam1 (n=12)	Dam2 (n=13)	Dam3 (n=9)	Dam4 (n=11)	Dam1 (n=12)	Dam2 (n=8)	Dam3 (n=12)	Dam4 (n=11)
Brain	0	0	0	0	0	0	0	0
Lungs	0	0	0	0	0	0	0	0
Heart	0	0	0	0	0	0	0	0
Liver	0	0	0	0	0	0	0	0
Kidney	0	0	0	2 (n=1) 0(n=10)	0	0	0	0

4.0 Discussion

Studies evaluating the pharmacokinetics of 17-OHPC in rats after different routes of administration ruled out the oral route since it exhibited poor bioavailability. This may be due to poor absorption, efflux or metabolism of 17-OHPC in the gut. Hence, the next route explored was the vaginal route.

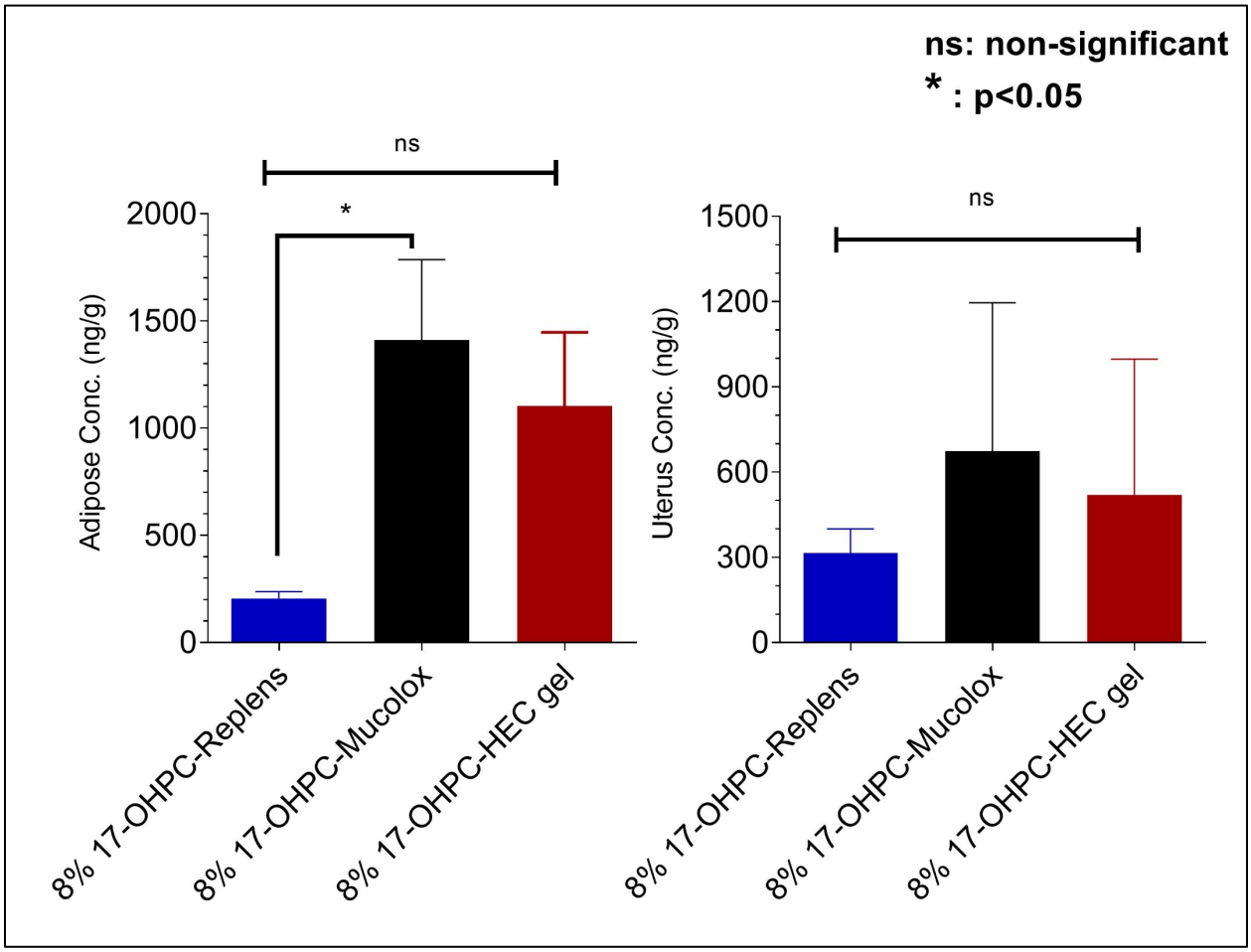

Figure 14: Tissue levels after administration of different 17-OHPC gels

The objective of the current study was to evaluate the safety of 17-OHPC vaginal gel in pregnant rats since preliminary data from our lab has shown that the 17-OHPC intravaginal gel does not cause any toxicity in non-pregnant rats and rabbits. This is the first study to evaluate the safety of intravaginal administration of 17-OHPC which is the only drug available for the prevention of preterm delivery.

Previously, 17-OHPC vaginal gel was prepared in three bases viz. HEC, replens and mucolox. Short-term safety studies have been done with these gels in non-pregnant rats and rabbits and safety has been established. Replens gel had problems with leakage in the rabbits and we anticipated that this may lead to non-uniform dosage and hence decided to use HEC and mucolox gel for further studies.

A 8 % progesterone gel (Crinone®) is used in the clinic for prevention of preterm birth in women with short cervix. Since the mechanism and site of action of 17-OHPC is still unknown we anticipate that creating a high local exposure like progesterone near the site of application may lead to a greater benefit. Hence, we decided to conduct further studies with mucolox base since it showed the highest concentrations of 17-OHPC near the site of application in the uterus and surrounding adipose tissue. We decided to use a 8% gel of 17-OHPC similar to the clinically used progesterone gel.

As shown in figure 10, mucolox gel showed highest release in the uterus and surrounding adipose tissue. Kruskal-Wallis test with multiple comparisons was used to compare 17-OHPC concentrations in uterus and adipose tissues from different gels at a p-value of <0.05 being significant. The results are presented as mean ± SEM. 17-OHPC concentrations in adipose were significantly different between the replens and mucolox gel (p-value of 0.0324), but not significantly different between the other groups (p-value > 0.99). 17-OHPC concentrations in the

uterus were not significantly different between the gels (p-value of >0.99). 17-OHPC levels in the plasma and ovary were below the limits of detection of 1 ng/ml.

Preliminary unpublished data from the lab has established that the mucolox gel with 8% 17-OHPC is stable for at least a period of 6 months. The histopathology assessment of vital organs from the mother indicated that the intravaginal application of 17-OHPC causes no damage to the tissues from the mothers. No toxicity was seen in vital organs of the pups from control and treated mothers. 17-OHPC intravaginal gel is safe in pups delivered to control as well as treated mothers. A high local exposure was seen in the uterus and surrounding adipose tissue. The 17-OHPC levels in the ovary were below limit of detection. No systemic exposure was observed after intravaginal application of this gel. In conclusion, the results indicate that 17-OHPC is not teratogenic in pregnant rats and does not cause any malformations in the delivered pups. Thus, a 8% 17-OHPC mucolox gel appears to be suitable for further evaluation.

5.0 Future Directions

We are currently evaluating the in-vitro release of 17-OHPC from the developed gel. We have developed and validated a method of measuring 17-OHPC concentration in simulated vaginal fluid using HPLC-UV. We plan to evaluate in-vitro release of 17-OHPC from the mucolox gel using the enhancer cell apparatus in simulated vaginal fluid. In-vitro release studies will give us an estimate about whether the drug needs to be dosed once a day or multiple times a day.

One observation during this study was that histopathology scores of 2 or 3 were obtained for the vagina and uterus tissue with intravaginal application of the gel in the control group. Additional studies are needed to determine if these observations are due to the insertion of applicator into the vagina. Another comparison group that receive intravaginal progesterone can be added to compare regional distribution in uterus and cervix.

Having established safety in pregnant rats, we plan to do a long-term safety evaluation in rabbits in the future since FDA requires safety studies to be conducted in at least two animal species (one rodent and one non-rodent). We plan to do a preliminary safety evaluation of the intravaginal gel in post-menopausal women in the future before conducting clinical studies in pregnant women.

The ultimate goal of the project is to develop an alternate formulation to the currently available IM injection which is safe and effective and thereby improve outcomes in pregnancy.

Appendix A

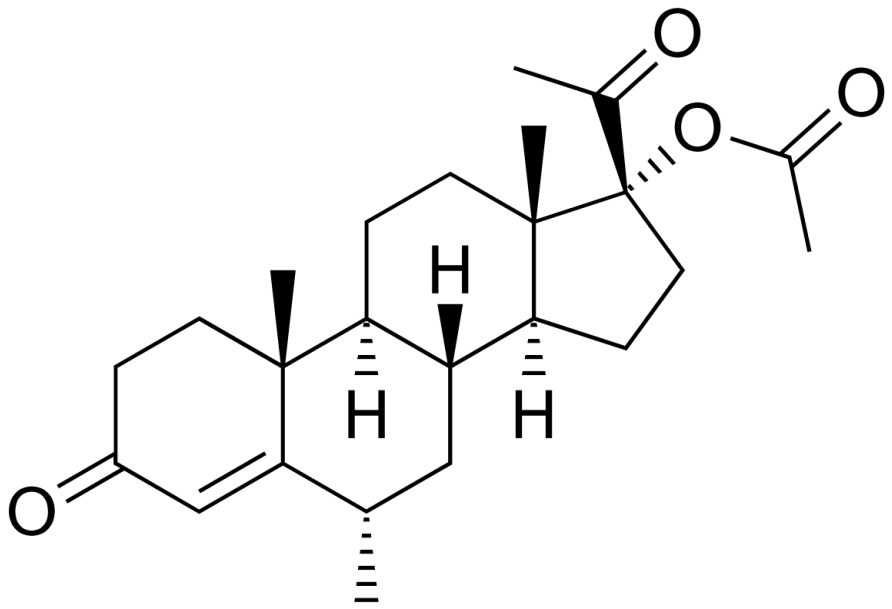

Figure 1: Medroxyprogesterone acetate

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