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Cell Biomechanics of Cervical Fibroblasts for Studies of Cervical Ripening in Preparation for Birth

Thesis
Presented in partial fulfillment of the requirement for the graduation of research distinction with a degree in Biomedical Engineering in the College of Engineering of The Ohio State University.

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Abstract

Preterm birth (PTB, delivery <37 weeks gestation) is the leading cause of mortality and morbidity in infants less than one year of age, yet the mechanism for preterm birth is not well understood. It is evident that inflammation of the cervix is a proximate cause for preterm labor and that progesterone is essential for maintenance of normal pregnancy. This study aimed to better understand the role of progesterone in the biomechanical changes that occur in cervical fibroblasts during labor and cervical ripening. To do so, collagen type I contraction assays were used as cellular models to determine the effects of progesterone and cytokines on collagen ECM remodeling) contraction. Cervical fibroblasts were seeded into collagen type I gels and treated with cytokines (Interleukin 1 Beta (IL-1β), Transforming growth factor beta (TGF-β), or Tumor Necrosis Factor alpha (TNF-α)), progesterone, or both at either 0.2 ng/mL or 2.0 ng/mL for seven days. The change in area of the gels was measured daily with image software ImageJ, to determine the effects of the treatments on the contraction efforts of the fibroblasts. Initial) results of this study indicate that the presence of progesterone enhanced the contractility of the collagen gels. These data suggest that progesterone does in fact influence the mechanical changes of the cervical cells and that it does not negate the effects of the inflammatory cytokines. These studies are the first attempt to examine how cytokines and progesterone can influence the fibroblasts as preparation for labor and birth occurs. With progesterone being a current treatment method for PTB. By

better understanding the mechanism behind preterm birth and the role of progesterone we can begin to devise a strategy to delay cervical ripening and pre term birth.

Acknowledgements

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Introduction

Preterm birth (PTB) is defined as delivery before 37 weeks gestation and is currently the leading cause of mortality and morbidity in infants less than one year of age. This medical anomaly is responsible for 30 billion dollars in annual health care expenditures in addition to the high cost associated with high risk births and complicated postpartum care [1,2]. The exact mechanism and reasons for PTB are not yet known nor well understood. However, it is known that cervical ripening, which leads to birth, is often caused by infection of the uterus which results in the increased production of cytokines and prostaglandins [3]. In addition to the inflammatory response, it has also been show that progesterone is essential for the maintenance of normal pregnancy including cervical competence [4,5].

If cervical ripening and birth occurs before 37 weeks gestation, the child is born prematurely putting both the infant and mother at great medical risk. As such, it is of high importance to determine the mechanism behind PTB in order to better refine current treatment methods. Doing so may allow medical professionals to prolong the gestational period for women experiencing early signs of PTB, decreasing the risk of the pregnancy and promoting the health of the child. In addition to increase in health of the mother and child, this would also decrease the astronomical annual medical expenditures associated with caring for those affected by PTB.

This project examined how progesterone mediates the mechanical changes that occur in the cervix associated with labor. Specifically, we hypothesized progesterone would act as a potential inhibitor of the contractile response of the cervical cells due to the increase in cytokines, which is often associated as a proximate cause of the onset of PTB. This study was the first attempt in the literature to examine how cytokines and progesterone can influence the cervical fibroblasts as preparation for labor and birth occurs.

Methodology

Cell Culture and Preparation

Primary cervical stromal fibroblasts were used in this study and were cultured according to the model developed in the Kniss lab [6]. These cells were isolated and cultured from premenopausal women who were undergoing hysterectomies for non-cancerous diseases. To begin, the cells were cultured in complete basal medium consisting of high-glucose (4.5 g/L) Dulbecco modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum, 50 μg/mL gentamicin sulfate and 0.5 μg/mL amphotericin B [7]. After reaching ~70% confluence, the media was switched to medium composed of phenol red-free DMEM/F12 (1:1) supplemented with 0.5% charcoal stripped FBS. In order to develop progesterone receptors, the cells received a supplement of 17β-estradiol (17β-E_2, 10^{-7}M/L) for 7 days [7]. After 7 days, the cells were then treated with Progesterone (P_4). This procedure was first developed in a study conducted by Ackerman et al. [7].

For this experiment, the behaviors of control cells, cells treated with only estrogen, and cells treated with estrogen and progesterone were compared.

Gel Preparation

Collagen type I contraction assays were conducted in order to model the effects of progesterone and cytokines on collagen contraction. A bovine type I collage contraction assay protocol similar to the one published by Cell BioLabs was followed [8]. To begin, the cells were harvested and re-suspended in the red free DMEM previously described at a concentration of 5×10^6 cells/ml. The following protocol is suggested for samples in a 24 well plate. To begin, the desired amount of 10x DMEM and collagen was added to a cold sterile tube in a 12:100 ratio respectively. Next, the 1 M NaOH was added to the sterile tube at a volume 10% of that of the volume of 10x DMEM previously added. This cold collagen gel solution mentioned (Collagen + 10x DMEM + 1 M NaOH) will account for ~3/4 of the total volume. Therefore, the remaining ¼ of the total volume will be the cells resuspendend in media. As an example, the volumes found in Table 1 below were used to prepare a total of 8 gels in a 24 well plate.

Table 1: This table details the reagents used and their quantities in order to produce a collagen gel solution sufficient to make 8 collagen gels in a 24 well plate.

Reagents	**8 wells**
Collagen Solution	2.58 mL
10x DMEM	320 μL
NaOH	32 μL
Cells resuspended in media	0.59 mL
Total	**3.792 mL**

All reagents were kept on ice for the entire gel preparation increase the time to gelation. After gently mixing the described collagen gel solution, approximately 400 μL of the solution were added to each well of a 24-well plate. After an hour, the gels were released from the sides of the well plate by sliding a pipet tip around its circumference. Afterwards, a milliliter of media was added gently to the top of each gel.

After 24 hours, the medium was removed and each well was treated with one of three different cytokines commonly seem in the cervix during labor. Each well received either 0.2 or 2.0 ng/mL of Interleukin 1 Beta (IL-1β), Transforming growth factor beta (TGF-β), or Tumor Necrosis Factor alpha (TNF-α). IL-1β and TNF-α were both chosen for this study as intraamniotic infusions of these cytokines have been suggested to induce preterm labor in other studies [9]. TGF-β was selected as a cytokine of study as during dilation/labor there is an increase in transcription of TGF-β responsive genes suggesting it may oppose the action of progesterone [10].

For this study, cervical fibroblasts were used as the control (CX) and compared to the responses of cervical fibroblasts treated with 17β-estradiol ($CX + E_2$) or cervical fibroblasts treated with 17β-estradiol and progesterone ($CX + E_2 + P_4$). Within each cell type, multiple wells were treated with either 0.2 or 2 ng/mL of Interleukin 1 Beta (IL-1β), Transforming growth factor beta (TGF-β), or Tumor Necrosis Factor alpha (TNF-α).

Imaging and Analysis

Following the cytokine treatments and release of the gels, the well plates were photographed at 6 hours and then again daily for the next 7 days. The area of the gels was then measured via analysis of the photos using ImageJ software.

Results

After obtaining the gel diameters, MATLAB was used to create contraction profiles of the various collagen gels and treatments. Figure 1 below presents the contraction profile of all three cell types, CX, CX+E_2, and CX+E_2+P_4, which were treated with 2 ng/mL of IL-1β.

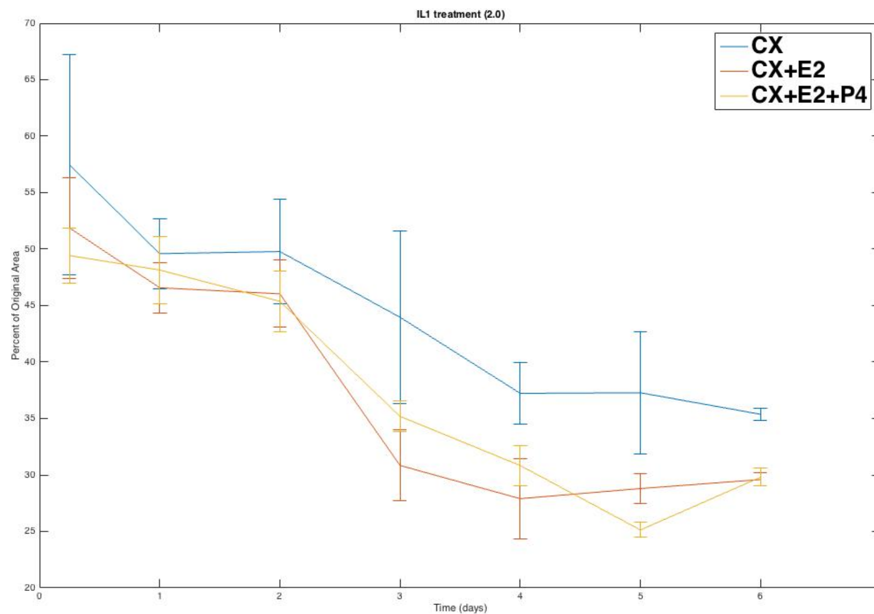

Figure 1: Contraction profile of three different cell environments (hormones or no hormones) treated with 2 ng/mL of IL-1β. The concentration profile is presented as plot relating percent of original gel diameter vs. time.

Figure 2 below presents the contraction profile of all three cell types treated with 2.0 ng/mL of TGF-β.

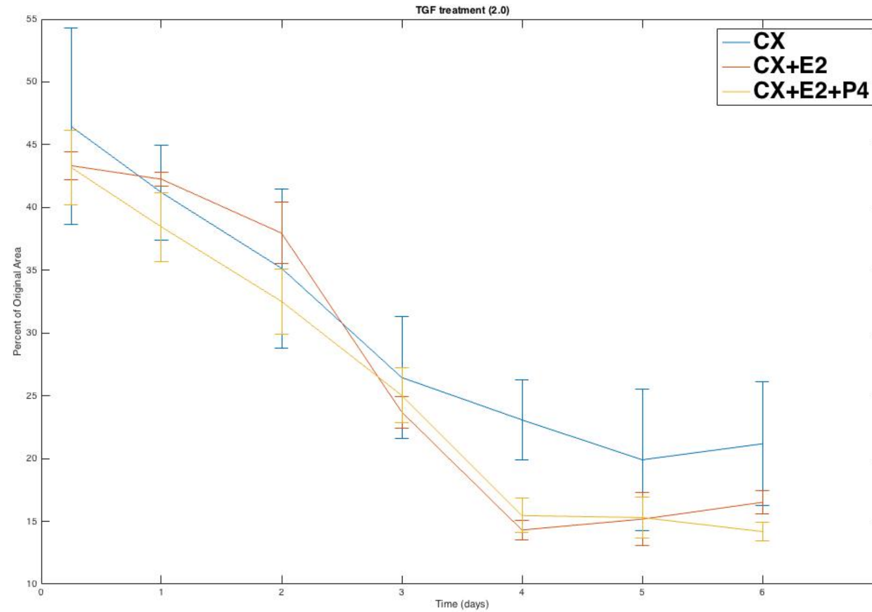

Figure 2: Contraction profile of three different cell environments (hormones or no hormones) treated with 2.0 ng/mL of TGF-β. The concentration profile is presented as plot relating percent of original gel diameter vs. time.

Figure 3 on the following page presents the contraction profile of all three cell types treated with 2.0 ng/mL of TNF-α.

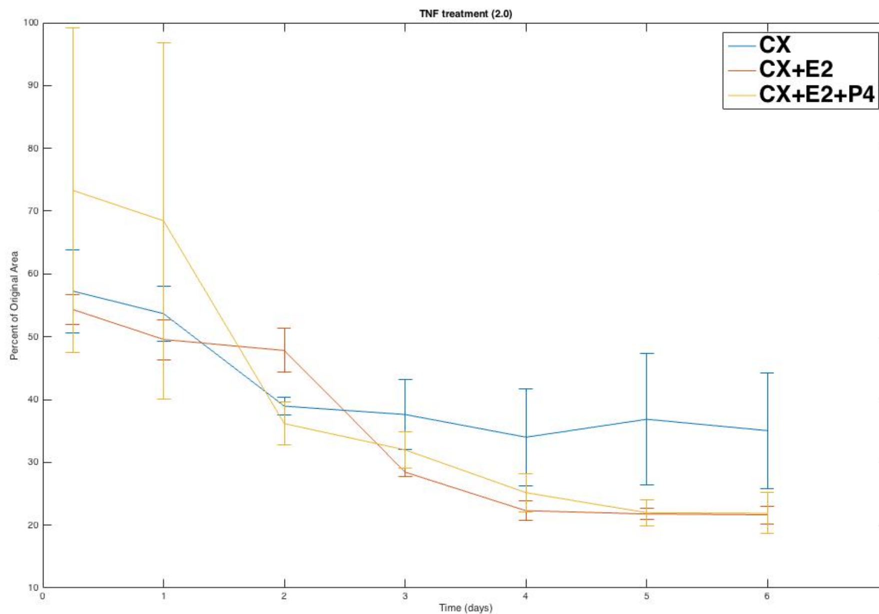

Figure 3: Contraction profile of three different cell environments (hormones or no hormones) treated with 2.0 ng/mL of TNF-α. The concentration profile is presented as plot relating percent of original gel diameter vs. time.

As demonstrated in Figures 1-3, the collagen gels in the presence of either estradiol (CX+E_2) or progesterone (CX+E_2 + P_4) contracted to a far smaller percentage of the original area than the collagen gels containing the cells that lacked the interaction with hormones (CX). These results indicate that progesterone in the presence of cytokines enhances the contractile response of the cervical fibroblasts. These results were contrary to our original hypothesis and suggest that progesterone can exert mechanical changes in cervical fibroblasts.

This trend is also seen in the cells that were not in the presence of any cytokines. Below in Figure 4, the contraction profile of the control cells also exhibited a greater

contraction when treated with hormones estrogen and progesterone.

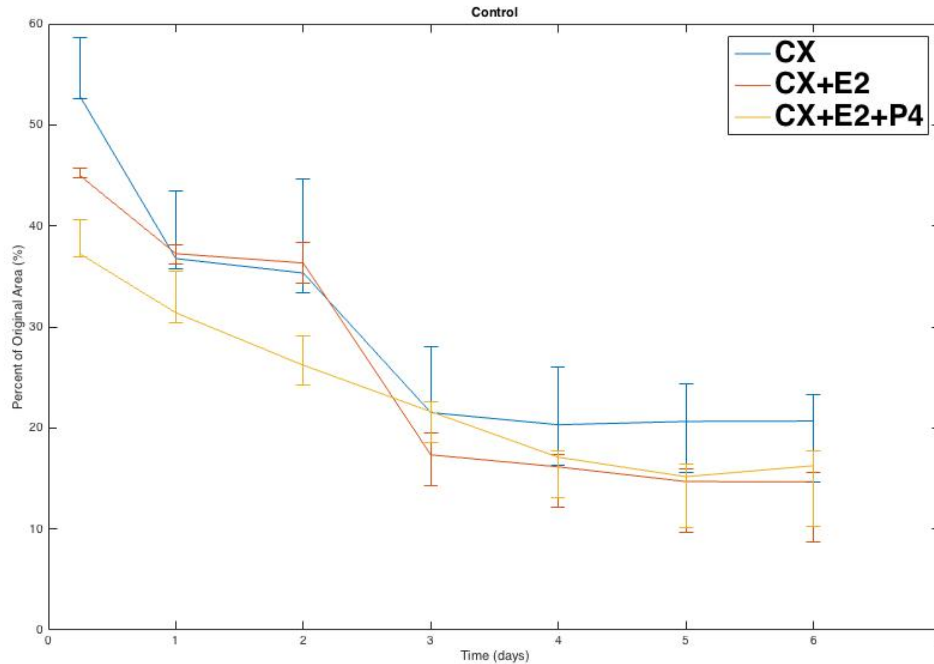

Figure 4: Contraction profile of three different cell environments (hormones or no hormones). These cells lacked the interaction with inflammatory cytokines yet exhibited a similar contraction profile as those in the presence of inflammatory cytokines.

Additional concentration profiles can be found in Appendix B.

Conclusion

With preterm birth accounting for a large medical expenditure with devastating affects to the woman and children involved, it is imperative we begin to better understand the mechanism behind it. This study allowed us to better qualify progesterone's role in the birthing mechanism. Although once thought of to be the maintainer of pregnancy and preventer of cervical ripening the results of this study suggest otherwise. The increase in contractility in the presence of progesterone implies that progesterone may elicit mechanical changes in cervical fibroblasts.

Treatments for PTB currently utilize progesterone as a means to slow contractions and prevent cervical ripening. However, based on the results of this study, this treatment may either be ineffective or counterintuitive.

In the future, this experiment will be repeated in order to generate more data to eliminate errors that could have arisen through the smaller sample size. Additionally, an experiment that better mimics the complexity of the cervix may better represent the contractile response of the cervical cells and progesterone's role in maintaining them. The cervix is a multi-layer, multi-cellular organ, where our experiment focused on a thin layer of a single cell type. As such, it may be beneficial for future experiments to broaden the scope of view to include a more 3-D model of the cells involved. Additionally, inclusion of more cell types and extracellular matrix components may develop a more accurate model representing the cervix during labor.

In sum, while repeat experiments are required, early results indicate progesterone enhances the contractile responses of the cervical fibroblasts in the presence of inflammatory cytokines. This study serves as an important tool in refining treatment methods to prevent pre-term birth, as progesterone is a current treatment used to delay labor. Assuming this model is correct, future studies may suggest progesterone is ineffective in this sense and suggest a more appropriate treatment method.

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Appendix A: Contraction Profile Data

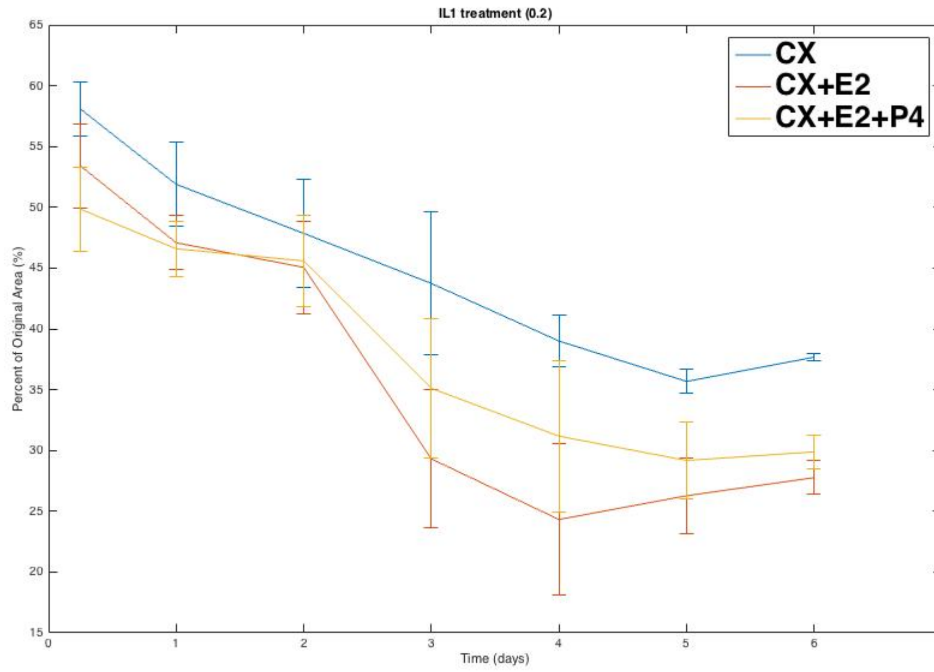

Figure A1: Contraction profile of cells in the presence of various hormones treated with 0.2 ng/mL of IL-1β.

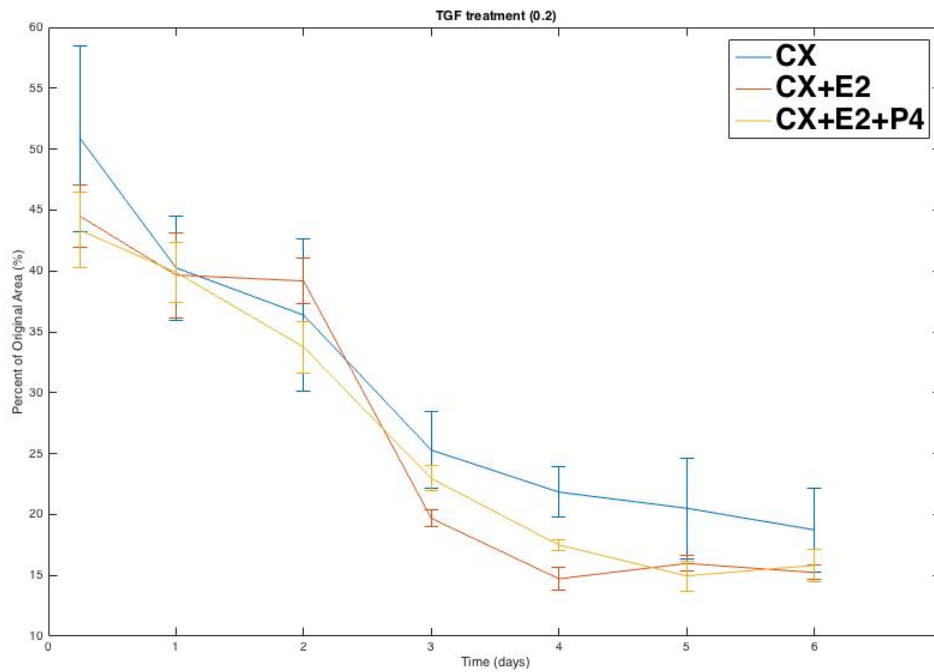

Figure A2: Contraction profile of cells in the presence of various hormones treated with 0.2 ng/mL of TGF-β.

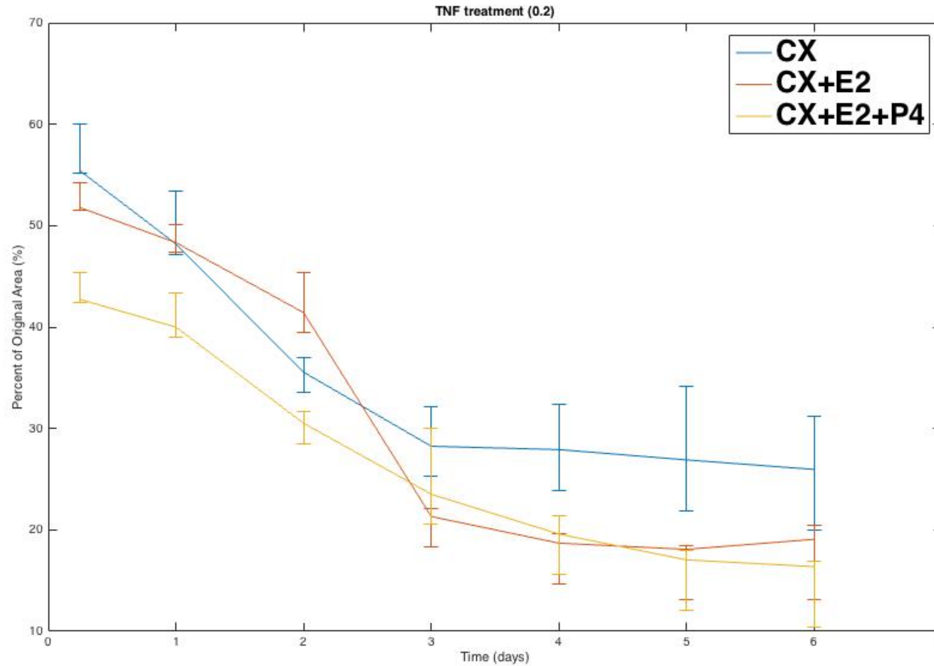

Figure A3: Contraction profile of cells in the presence of various hormones treated with 0.2 ng/mL of TNF-α.