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Effect of progesterone, terbutaline and leptin on the function of alveolar type II cells

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Effect of progesterone, terbutaline and leptin on the function of alveolar type II cells

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Master of Science

By
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Shamili Sammohi ENTITLED Effect of Progesterone, Terbutaline and Leptin on the function of alveolar type – II cells BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Shamili Sammohi, M.S. Department of Pharmacology and Toxicology, Wright State University, 2015. Effect of Progesterone, Terbutaline and Leptin on the function of alveolar type – II cells.

The "surfactant" produced by type II pneumonocytes is deficient in term and preterm infants born and diagnosed with Respiratory Distress Syndrome (RDS). Corticosteroids such as dexamethasone or betamethasone are clinically used as the primary line of treatment to stimulate the production of surfactant. The steroidal hormone, progesterone appears to play a role in the fetal lung development and also appears to increase the expression of inflammatory markers in both term and preterm infants. However, the impact of progesterone on surfactant production remains unknown. Like progesterone, the tocolytic drug terbutaline has also been implicated in phosphatidylcholine production in the pneumonocytes of the human lung. Interestingly, recent reports indicate that leptin, a hormone mainly produced by adipocytes may increase surfactant production *in-vitro*. However, other authors could not reproduce those results. This study was designed to analyze the long-term effect of progesterone, terbutaline and leptin on surfactant production *in-vitro*, either alone or in combination with betamethasone as a positive control. As a model, we used the human lung cell line NCI-H441. The production and processing of Surfactant Protein-B (SP-B), in this cell line is almost identical to explant cultures of fetal lung and cultured type II cells. Biochemical, immunochemical and molecular approaches, including thin layer chromatography (TLC) and RealTime-PCR

were followed to determine the pharmacological role of progesterone, terbutaline and leptin on surfactant production. Our experiments indicate that betamethasone increased lipid secretion and surfactant-B production at 24 hours. Likewise, progesterone and terbutaline, increased lipid secretion and surfactant production when analyzed by immunocytochemistry. Betamethasone also increased surfactant production when added to these tocolytics. RealTime PCR also showed a similar increase in surfactant-B mRNA. The results suggest that progesterone in combination with betamethasone may improve surfactant production in high preterm risk patients. However terbulatine and leptin need further studies on their mechanisms and future use.

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Introduction

Statement of the problem

This study is about specific drugs that are used to treat pregnant women who are at a risk of delivering their babies before their due dates. According to the World Health Organization (WHO), preterm birth is defined as the birth prior to 37 complete weeks of gestation. Based upon the gestational age of the infant, preterm birth is further subdivided into three categories as; 1) extremely preterm, for infants who are born before 28 weeks of gestation; 2) very preterm, i.e., 28 – 32 weeks of gestation; and 3) moderate preterm for infants born between 32 – 37 weeks of gestation (1). Although there are numerous problems associated to the preterm birth, one of the most common and critical problem is Respiratory Distress Syndrome (RDS) (2). Infact more than 60% of the preterm births are seen in Africa and South Asia and 12% of infants are born prematurely when compared to higher income countries (1). For instance, in developed countries such as the US and UK, premature births along with RDS occur in 1 – 2 % of the total pregnancies each year (2, 5). In contrast, developing countries such as India and Pakistan have a much higher rates of premature infant births of 10 – 50% (3, 4). The risk of neurosensory disability and risk of death escalates, with a decrease in gestational age (especially infants born between 26-28 weeks of gestation) (5, 6, 7). A reliable diagnostic tool to predict the risk of preterm birth is not yet available. Therefore, the main treatment has been to try to delay birth for up to 48 hours, which would allow time to administer specific drugs to

help mature the lungs and prevent RDS. To delay birth, there are several drugs that act to slow the contractions of the uterus and delay preterm birth for 1 or 2 days. This additional time is often sufficient for the administration of corticosteroids, which helps in the maturation of lungs potentially allowing the premature infant to breathe properly soon after its birth. However, scientific studies have not been carried out on the effect that these drugs have on surfactant and lipid production in lung cells. This study was designed to determine if these drugs have an impact on lipid and surfactant production in lung cells.

Respiratory distress syndrome

RDS in preterm infants is characterized by a lack of surfactant. Without enough surfactant, the lungs collapse and the infant has to work harder to breathe (8). The infant might not be able to breathe in enough oxygen to support the body's organs and the lack of oxygen (hypoxic condition) may cause damage to the brain and other organs if proper treatment is not provided immediately. The primary treatment when a preterm birth is likely is to give dexamethasone or betamethasone to accelerate the expression of surfactant (9, 10, 11). The duration of treatment can be for 24 hours or 48 hours depending upon the type of corticosteroid prescribed. The common regimen recommended by the NIH Consensus Development Panel (9) in 1995 for betamethasone was; 2 doses of 12 mg given intramuscularly 24 hours apart and 6mg of dexamethasone given intramuscularly in 4 doses 12 hours apart for each dose. In-order to determine if this dose is high enough to cause adverse effects (10), the NIH panel in 2000 met again after randomized control trials were performed at different regions of the world and

concluded that there was no evidence for the adverse effects of the drug and suggested to continue the same regime for 7 days for those who are at risk for preterm delivery (9, 10).

Type II alveolar cells, especially pneumonocytes are very specialized cells that produce and secrete lipids and surfactant, which are responsible for maintaining the stability and function of the lungs. During the first breath of the infant, a considerable force is necessary to overcome the high viscosity and inertia of the fetal lung fluid that is filled in the alveolar sacs during birth (8). Due to the high pressures generated by the first breath, they aid in moving the lung's fluid from the sacs and the formation of an air-liquid interface. As the air enters, the reduction in size of airways at the periphery causes an increase in surface tension at the air-liquid interface (8). Presence of an adequate surfactant maintains the stability of lungs by lowering the surface tension at the junction. As the alveolar surfactant is produced after 30 weeks of gestation, most of the preterm births are leading to RDS and finally to the death of some of those infants, due to the lack of surfactant production.

Surfactant

Surfactant is a form of 'detergent' encased into small cellular packets called lamellar bodies (13), mostly produced by specialized lung cells called Type II pneumonocytes. Surfactant helps lungs to inflate and deflate by lowering the surface tension on the cell surface inside the lung (12), thus aids in stabilizing the alveoli during respiration (15). Surfactant is synthesized as a complex mixture of lipoproteins containing 70-80% phospholipids, 8–10% protein, and 10% neutral lipids in which lecithin or dipalmitoyl phosphatidylcholine (DPPC), is the main phospholipid component (2). By predominantly possessing the phospholipid fraction, lecithin, the surfactant is

believed to be a lipoprotein (15). In addition, phosphatidylglycerol makes up 4–15% of the phospholipids, although it is not necessary for the lung function, but it has been used as a marker for detecting the fetal lung maturity (2).

Surfactant Production

The components of pulmonary surfactant are synthesized in the endoplasmic reticulum (ER) of Type II alveolar cells and transported through the Golgi apparatus to secretory vesicles where they are packed and stored in lamellar bodies (13, 14), as represented in figure 1 below. After these granules are discharged into the liquid lining of the alveolus, the surfactant phospholipids act to form a complex lattice called tubular myelin (13). The surfactant production is considered a cyclic process in which the phospholipid in the lumen is moved in and out of the Type II alveolar cell and reused up to 10 times before being degraded (2). The surfactant phospholipids and proteins are taken back subsequently into Type II cells, by an endosomal pathway in the form of small vesicles and are transported into lamellar bodies for storage and further recycling. After they are secreted, the lamellar bodies unwind to form bipolar monolayers of phospholipid molecules. This process depends upon two important apoproteins; SP-B and SP-C (13). Phospholipid provides the material which is used to form a monolayer at the air-liquid interphase to lower the surface tension inside the alveolar type – II cells, which is produced from tubular myelin. The tubular myelin depends on surfactant-B for performing its functions and stores surfactant in it.

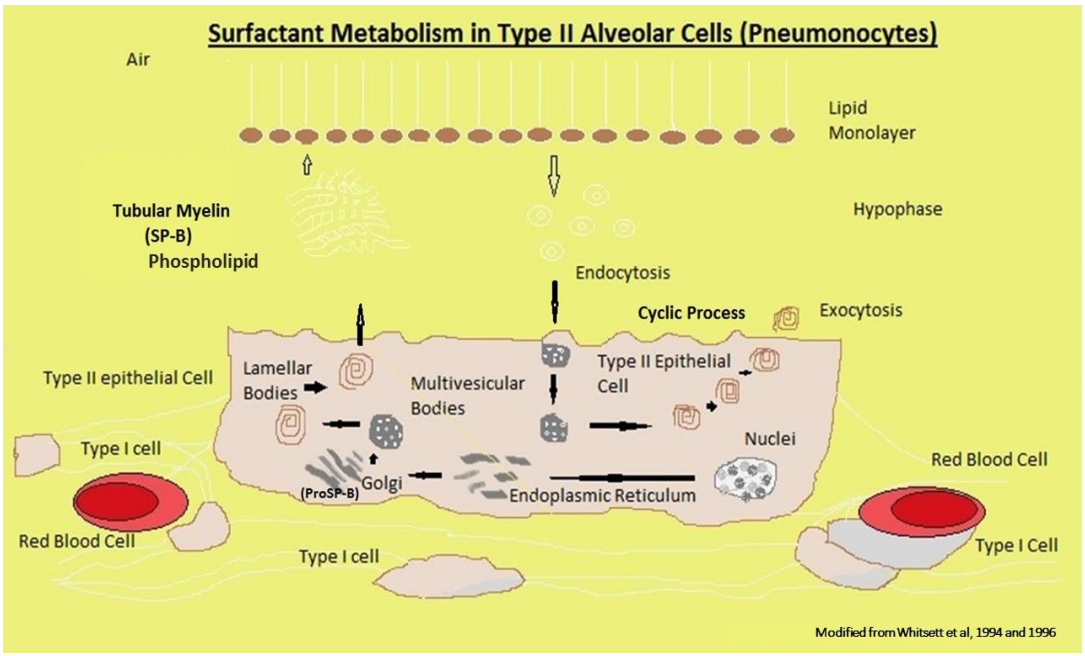


Figure 1: Surfactant metabolism in type II alveolar cells (Pneumonocytes) which has been modified from Whitsett et al., 1994 and 1996 (16, 17, 20)

Surfactant proteins B and C (SP-B and SP-C)

There are four surfactant proteins produced in the lungs: Surfactant Protein–A (SP-A), Surfactant Protein-B (SP-B), Surfactant Protein–C (SP-C) and Surfactant Protein–D (SP-D). Among these, hydrophilic proteins, SP-A and SP-D possess considerable similarities in the sequence of their proteins (81) and also play a role in innate immune responses especially inside the lung (82). On the other hand SP-B and SP-C proteins are similar in nature and function (18, 19, 20). Surfactant protein B (SP-B) and SP-C are small hydrophobic proteins (9 kDa) that are responsible for 2-4% of the surfactant mass (18, 19). SP-B starts as a ~40 kDa pro-protein that is cleaved to become a ~9-kd protein in the type II alveolar cells just before entering the lamellar bodies (20, 19).

SP-B has been shown to mediate rapid absorption and spreading of DPCC as a monolayer to the air- liquid (alveolar air-fluid) interface in vivo during expiration (18).

Role of Surfactant Protein – B in Type II Cells

It has been shown that the activity of SP-B is necessary for rapid film formation by phospholipid mixtures. Also, that even though the surfactant consists of both hydrophilic and hydrophobic proteins, it is the hydrophobic proteins that most likely have a major role in determining the surface-active properties of the surfactant (21). SP-B and the phospholipid dipalmitoylphosphatidylcholine (DPPC), commonly known as lecithin, helps to lower the surface tension of the lung so that breathing can occur (18). Secretion of the pulmonary surfactant by type II pneumonocytes is considered to be a rate-limiting step in delivering the surfactant to the air-liquid interface which eventually helps in lowering the surface tension inside the lung, which largely depends on SP-B (18).

Importance of maintaining surface tension in alveolar type II cells

In most of the species, certain lining cells of the terminal respiratory units undergo alterations allowing them to be distinguished as Type II pneumonocytes during the last 10-20% of gestation (22-26 weeks of gestation) (22). Due to the absence of surface tension lowering surfactant, alveoli become unstable at low volume and tend to collapse. In addition, some of the smaller alveoli get emptied into larger ones to equalize pressure between two of them, which may even be dangerous for the newborn to breathe properly. The high pressures generated in the first few breaths of the newborn are not only required for expansion, but also crucial in moving the lung liquid for establishing an

air-liquid interface (23). During this process, the surface tension at the junction of lung fluid with air increases and the clearance of fluid from smaller airways may be more efficient in the presence of adequate surfactant. Due to the lack of an adequate amount of surfactant, newborns with RDS are highly prone to death.

Phospholipids (Lecithin/DPCC and Sphingomyelin) and their ability to predict RDS

The lung is known to be an active site of lipid synthesis due to the process of incorporation of acetate into fatty acids and phospholipids. Lecithin, i.e., phosphatidylcholine, is the major fatty acid that is incorporated into phospholipids (24). The amount of lecithin and the proportion of palmitic acid are increased dramatically in the fetal lung along with gestational age, suggesting that these increases could be closely related to the development of surface activity of the lung. Sphingomyelin is also a phospholipid that aids in surfactant production. An alveolar lecithin/sphingomyelin ratio of less than 2 is a reliable predictor of RDS in premature infants and a noticeable increase in lecithin content of the fetal lung is considered as a characteristic feature of pulmonary maturation (25). Fetal lung maturity is measured by counting the lamellar bodies. At ~32-33 weeks of gestation, the concentration of lecithin in the amniotic fluid begins to escalate almost constantly until term, whereas the concentration of sphingomyelin remains relatively constant and hence, the ratio of these lipids, lecithin and sphingomyelin provides an estimation of the amount of surfactant being produced (26, 27). Therefore, the prevailing dogma is that RDS is most often due to a developmental deficiency of pulmonary surfactant along with an immature lung function, which is similar to the condition of premature birth where all the surfactant components are

decreased (28, 29). This is not unexpected as fetuses do not normally have a need for a mature lung while *in utero*, as it is very early for the birth of infant.

Analyzing SP-B protein for an overall surfactant increase

SP-B plays a critical role in the formation of lamellar bodies within Type II cells (30), which are the absolute requirements for processing, storage, secretion and normal function *in-vivo* (31) and to facilitate the fusion of multivesicular bodies, also by assisting with a proper assembly of the lamellar structure of lipids. These are the fundamental reasons for selecting SP-B as the key protein for estimating the surfactant production in this project. Surfactant-B is a 10-kb SP-B gene which is located on chromosome 2, that encodes a 2.2-kb messenger RNA (mRNA) gives rise to 40-kD pre-pro SP-B (20, 19) and which is processed by both the amino and carboxyl regions to result an 8-kD mature SP-B. This mature SP-B is highly lipophilic and is packaged along with surfactant phospholipids in the lamellar bodies of alveolar type II cells (14). The absence of SP-B would lead to an accumulation of multivesicular bodies along with the formation of disorganized and unfinished lamellar structures, which further aids in producing membranous vesicles (29). This defective formation and secretion of lamellar bodies in the absence of SP-B can also delay the normal accumulation of phospholipid and surfactant proteins in amniotic fluid according to a reported case study of Hamvas, A et al (32).

NCI-H441 cells

Aside from primary cells cultured from living tissue, there are no fetal or infant lung cell lines available for this type of research. Primary cells from fetuses and infants

are also not readily available. Therefore, we have used the human adenocarcinoma NCI-H441 cell line obtained from ATCC, which has been used for other similar projects. Vidaeff et al (33) has suggested that the NCI-H441 cell line has been very useful for investigating the regulation of surfactant components in previous studies on dexamethasone over extended periods of time frame, i.e. 7-8 days. The synthesis and processing of SP-B in this cell line has been reported to be very similar to the explant cultures of fetal lung and also to the cultured isolated type II cells (34). Hence, the H441 cell line has proven to be useful for studying the surfactant protein expression and production of these proteins, which are of importance clinically in the diagnosis and therapy of various pulmonary disorders. In a study screening 33 human lung adenocarcinomas, only NCI-H441 cells were found to synthesize and secrete both SP-B in significant abundance, which were not detected in the A549 adenocarcinoma line (34). These cells were also shown to respond to the multiple regulatory factors that influence SP-B expression in a similar manner as observed in human type II pneumonocytes (35).

Betamethasone (Positive control in this study)

Corticosteroids, i.e., dexamethasone and betamethasone, have been shown to play a role in stimulating the synthesis of surfactant in the alveolar Type II cells *in-vivo* in the fetal lamb (36) and are still the most popularly used corticosteroids in clinical practice today for stimulating the surfactant production and lung metabolism. Although there is similar efficacy of both of dexamethasone and betamethasone on reducing the risk of RDS, the reduction in mortality rate was significantly higher with betamethasone versus dexamethasone (48% vs 11%) (37). The maximum stimulatory effect of fetal lung with betamethasone has been observed at 10^{-7} mol/L (35), which is the same concentration we

used in our study. The glucocorticoids (GC) usually binds at the glucocorticoid receptor (GR), although certain areas of the receptor shows a fundamental similarity with other steroidal hormones for their easy binding (38). Glucocorticoid responsive elements (GRE's) are present in the promotor regions of steroid-responsive genes (39, 67). It is believed to have both positive responses, i.e., transcriptional activation of the gene and negative responses, i.e., causes suppression of the gene with this binding at particular regions of the gene (38). This process is mediated by direct binding of GC-GR complex to GRE, which results to show some conformational changes in the DNA. The interaction of this complex also increases binding of other transcriptional factors such as activating protein-1 or nuclear factor kappa- beta (NFK- β) (40). In a premature rabbit model of human RDS, combined therapy with antenatal corticosteroids and intratracheal natural surfactant has given a superior result than the therapy alone with corticosteroids (41). Our study is designed to test some of these drugs with betamethasone.

Tocolytics

Tocolytics are the most common medication used to suppress or delay the preterm labor by causing anti-contractions of the uterine muscle. The therapy also gives time for the administration of betamethasone, which greatly accelerates fetal lung maturity, but takes one to two days to work. We have chosen two drugs that are often used to delay contractions and mature lungs in conjugation with betamethasone. The two drugs selected for this reason are progesterone and terbutaline. In addition, we are also interested to observe the effect drug leptin may have on the lungs, as these are the ports of leptin receptor in the fetal lungs. Also, to observe the effects of this peptide hormone, if it plays a role in the metabolism and secretion of lipids or surfactant.

Progesterone

Progesterone is a steroidal hormone supplied by the placenta during fetal development. Previous studies on the expression of progesterone and estrogen receptors have indicated that both of these may play a role in fetal lung development (43, 44). This is achieved by the suppression of calcium-calmodulin-myosin light chain kinase system through a process of reducing the calcium influx into the epithelial membrane and altering the resting potential of smooth muscle (45). The combined use of 17-β Estradiol and progesterone has shown to enhance the expression of surfactant proteins involved in the primary embryonic lung cells (43). The supply of these hormones are shown to be disrupted in the extreme preterm infant when compared to the term infant (42), which gives an impression that the lack of supply of hormone progesterone, which is necessary for maintenance of lung functioning and uterine contractions in preterm fetus may be an important factor for causing preterm birth. This led us to a new way of investigating the presence of progesterone in alveolar type II cells for the secretion and production of surfactant and also phospholipids when treated alone or in combination with betamethasone.

Terbutaline sulfate

Terbutaline sulfate, a β-2 adrenergic receptor agonist is used to treat patients suffering from asthma and some other respiratory diseases. Terbutaline sulfate has been shown to slow or attenuate contractions of the uterus, and thus is used as a tocolytic to delay the preterm birth (47). However, due to some adverse effects like increase in the maternal heart rate, changes in maternal diastolic blood pressure from the baseline, intraventricular hemorrhage and slight trauma observed by using this drug (71), its use

has been limited to 24 – 48 hours to delay the preterm birth according to the regulations provided by the US and the Institute of Medicine (US). Keeping those guidelines in view, we have chosen this drug to measure the surfactant increase in this study only for 24 – 48hrs of drug treatment. β- Adrenergic receptor agonists aid breathing problems by raising the levels of intracellular cyclic AMP (48, 49) causing an increase in the surfactant secretion by type II cells *in-vitro*. It has been already shown that terbutaline along with 3- isobutyl-1-methylxanthine (IBMX) stimulated surfactant secretion from alveolar type II cells. It has also been shown that these drugs increase the secretion of one of the major lipids responsible for the surfactant production, phosphatidylcholine (also called as lecithin) (50). It has also been approved that this drug terbutaline sulfate could be tested for the secretion of surfactant *in-vitro* and also *in-vivo* (51). Terbutaline has been studied to be efficacious and a more reliable drug for treating preterm labor than some of the drugs like nifedipine and salbutamol (72). Hence, one of the goals of this project was to examine the lung cells response to terbutaline sulfate either alone or in combination with betamethasone for determining its role in surfactant or lipid production and secretion.

Leptin

The peptide hormone, leptin is produced by the adipose tissue and placental trophoblast and has been suggested to regulate various aspects of human fetal development (52). It is believed that leptin acts through both autocrine and/or paracrine mechanisms, and it may also be an important factor in the maintenance of pregnancy itself (53, 54). Leptin binds to the leptin receptor (Lep_{rec}) which is present in the lung epithelium during antenatal and neonatal development periods, as it is present in many

tissues (55). The specific receptor for leptin is a member of the cytokine class I receptor superfamily, which is found to possess multiple existing isoforms (56). Leptin binds the Lep-R isoform that is present in the epithelium of type-II pneumonocytes and thus aids in inducing the expression of the major pulmonary surfactant proteins SP-A, SP-B and SP-C which are responsible for surfactant production (57). A shorter isoform (LEP-R_s) of leptin which is predominantly found in the peripheral tissues, such as lung, helps in contributing to leptin signaling by utilizing a mitogen-activated protein kinase (MAP-K) (58). Recently, animal studies have shown that leptin may also be involved in the regulation of the embryonic lung growth and maturation. In addition, antenatal administration of leptin resulted in an increase in the number and size of pulmonary cells in the newborn, which indicates that there is also an improvement in the development of lungs by enlarging the alveolar size and surface area (18, 59). Furthermore, leptin and its receptor have been identified in the type II pneumonocytes of fetal mice and have been co-related with an increase in production of surfactant-related protein in these cells when they were stimulated with leptin externally (60). From these experiments, a high level expression of the functional leptin receptor and also the detection of its splice variants in the fetal lungs altogether suggests that the specific binding for leptin in these cells (53, 60, 61). Some recent studies have indicated that a lack of exposure to leptin in late pregnancy, i.e., the point at which type-II alveolar epithelial cells mature and produce surfactant, would be subsequently responsible for contributing to RDS and Fetal Growth Restriction (18, 62).

Previous studies have shown that administration of betamethasone to these type of cells aid in upregulating the putative fibroblast pneumocyte factor in the lung

concomitant with an increase in lecithin in preterm infants (63, 64, smith 65) and also an up-regulation of placental leptin receptor protein in pregnant rat (66)

Development of Hypothesis:

Previous research has shown that betamethasone is a potent stimulator of lipid and surfactant secretion in alveolar type II cells. However, there are few studies showing a direct and combinational effects of tocolytics on surfactant and lipid secretion. The goal of this study was to identify the response of progesterone, terbutaline or leptin alone or in combination with betamethasone on human lung cells that secrete surfactant or lipid. To achieve this goal, we will test the hypothesis that betamethasone alone stimulates lipid production and secretion. Furthermore we propose that the tocolytics progesterone, terbutaline and peptide hormone leptin will also stimulate surfactant-B production and lipid secretion and that betamethasone will augment their effects. The hypothesis will be tested using immunochemical staining of cells for surfactant-B; thin layer chromatographic analysis of lipids and western blotting for cellular surfactant-B and leptin receptor protein. RealTime PCR analysis will be used to determine the effect of these drugs on surfactant-B expression at 24 hours and 48 hours following treatment.

Specific aims

Aim 1: To show that the administration of betamethasone causes an increase in surfactant production in human lung cells

Aim 2: Test the hypothesis that Terbutaline or progesterone increase surfactant production and lipid secretion from NCI-H441 cells

Aim 3: Test the hypothesis that leptin increases surfactant production and lipid secretion from NCI-H441 cells

Materials and methods

Experimental design

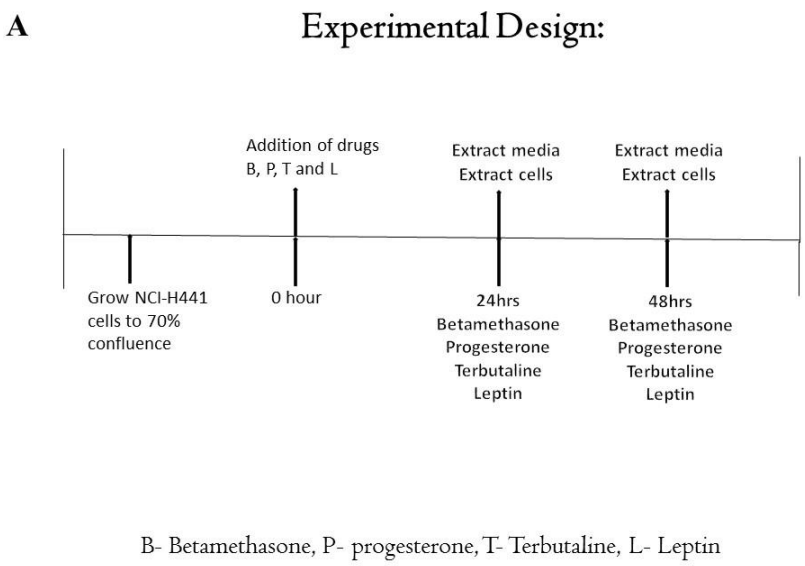


Figure 2: Experimental Design Part I

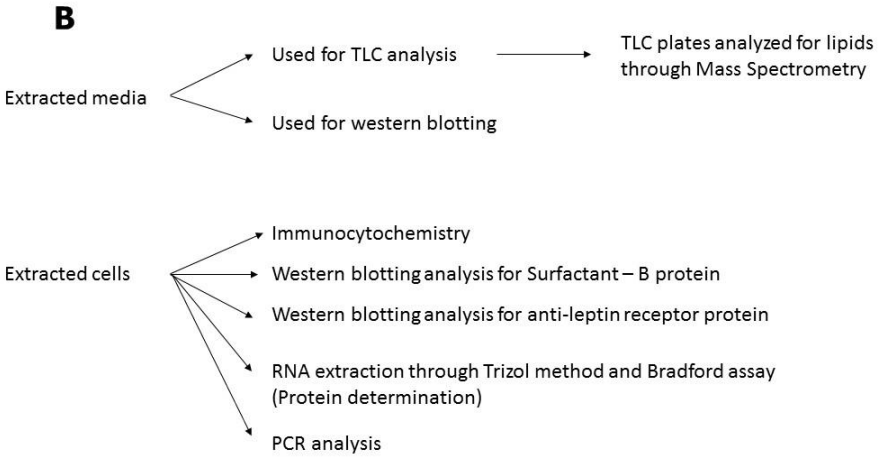


Figure 3: Experimental Design Part II

Table 1: Drugs and their concentrations used in the project

Drugs Used	Concentration
Betamethasone	10^{-7} M
Progesterone	10^{-6} M
Terbutaline	10^{-6} M
Leptin	0.1mg/ml

Table 2 above shows the list of drugs and their concentrations used to treat NCI-H441 adenocarcinoma cells used in this project.

Cell culture

The human lung cell line (NCI-H441) obtained from ATCC was originally derived from patient biopsy samples of a human adenocarcinoma. Since 1982 it has been extensively used as a transfection host for expression of pulmonary surfactant protein (32-34, 68). The cells were grown and incubated following the ATCC protocol. Briefly, NCI-H441 lung cells were grown in Waymouth's MB571/5 medium (Invitrogen) containing 10% charcoal-stripped FBS (Hyclone) and 1X antibiotic/antimycotic (Cellgro; containing 10,000 unit/ml penicillin G sodium, 10,000 µg/ml streptomycin sulfate, and 25 ug/ml amphotericin B). Cells were passaged by trypsin at 70% confluency to a new flask. After 8 passages (continuous multiplications) of the cells, all of them were trypsinized and grown into 4 different 12 well plates. After the cells became 70% confluent inside the wells, drugs were prepared according to their solubility and also the exact concentration of drug which showed a maximum potency according to clinical use.

As positive control, betamethasone was used at a concentration of 10^{-7}M, which is also the concentration used by the physicians to treat RDS or other preterm disorders. This drug was prepared by dissolving 3.92mg of betamethasone in 1ml of ethanol and

10µl of that solution was added to 990µl of 0.1%BSA (Bovine Serum Albumin) in Waymouth media. Then 50µl of the sterile drug was added into 49.95ml of media, which finally made it 10^{-7}M. BSA was added to replace the FBS (Fetal Bovine Serum). Progesterone was dissolved in ethanol. P8783 progesterone powder suitable for cell culture, was obtained from Sigma Life Sciences and dissolved with 1ml of absolute ethanol and 10µl of this solution was mixed with 990µl of 0.1% BSA in Waymouth media. Of this solution 500µl were added to 49.5ml of the media to finally make a 10^{-6}M solution. T2528 Terbutaline hemi-sulfate salt was also prepared following the same procedure except that it was dissolved in distilled water instead of ethanol. L4146 Leptin – 1mg human was dissolved in 1ml of distilled water and 1µl of this solution was added to 10ml of the 0.1%BSA which finally made a concentration of 0.1mg/ml. One well was left without adding any drug and in other wells 1ml of all these drugs have been added either individually or in combination with the positive control, betamethasone. After respective time intervals, the media was collected in centrifuge tubes separately and then 150µl of Tris EDTA (TE) buffer was added to the cells and collected into 500µl centrifuge tubes. All these tubes were neatly labelled and the cells were stored in -80°C and the media in -80°C. The collected cells and media were used for different experiments like HPTLC, Western blots and immunostaining.

The pictorial representation of the order of addition of different drugs can be seen in the image below.

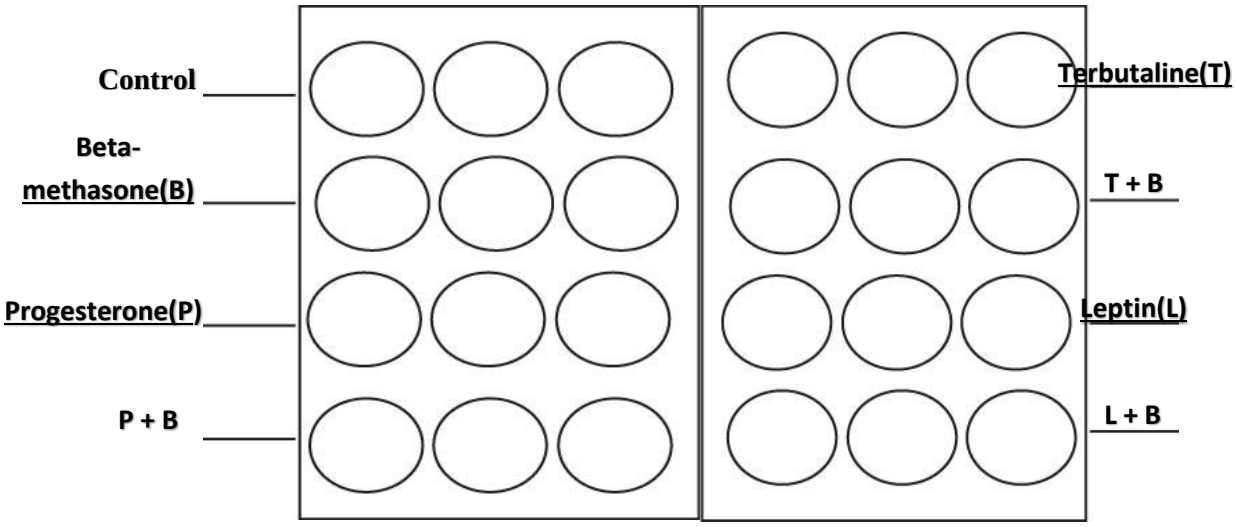


Figure 4: Plate scheme for all the experiments. The cell culture plates were set up in this order for all the experiments where cells were grown for TLC and western blots.

Thin layer chromatography (TLC)

High Performance Thin Layer Chromatography (HPTLC) is used to separate lipids on a solid phase based on the partition coefficient of lipids in organic solvents. This technique uses TLC plates with a more uniform and smaller particle size which will give a higher resolution of lipids with shorter retention times. The media and cells that were stored in the refrigerator were thawed and then 1ml of the media was added with an equal amount (1:1) of organic solvents 9:1 chloroform: methanol mixture. Then they were thoroughly mixed until the aqueous and organic layers separated. The lipids were soluble in the organic phase, hence the aqueous layer was discarded and the organic phase was dried by using speed vacuum overnight. After the lipids were mostly dried, 100µl of methanol was added to the lipids at the bottom of the tube and mixed very thoroughly in it. Standard sphingomyelin and lecithin (4µl) were spotted on the HPTLC Silica gel 60 F_{254} plates (Merck KGaA, Billerica, MA) and also TLC Silica gel 60 F_{254} plates (EMD,

Gibbstown, NJ) along with aliquots of 4µl of each sample. The lipids were resolved with an organic solvent system containing chloroform: methanol: acetic acid: acetone: ddH_2O at a 51:32:9:5:3 ratio. As we are already aware that the mobile phase flows through the stationary phase and carries the components of the mixture with it and different components travel at different rates. The spots were then seen clearly after the imaging. The stationary phase here is the silica gel or alumina, often contains a substance which fluoresces in UV light. When the solvent front has been reached ~0.5 cm from the top of the HPTLC plate, it was air-dried and sprayed with a mixture of primuline in water at a concentration of 1 mg/ml and again air-dried. The stock solution of primuline was prepared by dissolving 100 mg of primuline in 100 ml of water. The spray reagent was prepared by diluting 1ml of the stock solution in 100 ml of a mixture of acetone/ water (4/1, by volume). The primuline stained plates were examined under a DyLight 625 setting on the ChemiDoc MP imaging camera (Bio-Rad, Hercules, CA) for 5 seconds and the image was saved for evaluation. To analyze the bands produced on HPTLC plate, an ROI box was drawn around each band and the density was integrated for each band in each treatment. The integrated density was recorded and averaged for all three samples. Mass spectrometric analysis of the bands was inevitably required for confirmation of the band's true identity.

Immunocytochemistry

Cells were plated at a density of ~2000 cells/well in an 8 well Lab-Tek® slide chamber (Nunc, Naperville, IL) and treated with each of the drugs in the presence or absence of the positive control, betamethasone and one of the well was left as a control i.e. without addition of any drug including betamethasone. In order to compare the action

of these drugs upon the lung cells individually, some of the wells were administered with betamethasone alone and some with only these drugs to clearly differentiate the action of these drugs with and without the presence of betamethasone. The cells were fixed for 1 hour with 2% paraformaldehyde (2% PFA), (Electron Microscopy Sciences, Hatfield, PA) in Phosphate Buffered Saline (PBS). PFA (2%), was added with 0.1% Triton X100 i.e. 100 µl of Triton X100 in 10 ml of PBS after washing thoroughly with 1X PBS. The solution was removed and washed with 1X PBS 5 times for 5 minutes each. The cells were then incubated with 100 µl of Mouse Primary antibody, ab 3282 Ms mAb to Surfactant Protein B (Mature) [SPB02] obtained from Abcam, diluted at 1:250 in 5 ml of 10% goat serum overnight. The primary antibody was washed away 5 times with 1X PBS for every 5 minutes and the cells were then incubated with Donkey anti-Mouse IgG coupled to Cy3 [Cy 3- conjugated AffiniPure Donkey Anti-Mouse IgG (H+L)] obtained from Jackson ImmunoResearch Laboratories for 2 hours. The secondary antibody was washed with PBS. The cells were then washed once with deionized water and mounting was performed by using Prolong Gold Antifade reagent with DAPI obtained from life technologies (P36931) to stain the nuclei. Stained cells were visualized on a Leica DMR epifluoresecent microscope and images were captured using a Leica CCD digital camera system. These images were prepared for publication using Adobe Photoshop and Adobe Illustrator CC software. Non-stained cells were visualized on a light microscope.

Western blotting

The cells were lysed using a mini-pestle (twisted for about 10 times). This sample was taken into a fresh Eppendorf tube and added with 20µl of Laemmli Sample Buffer (Blue SDS Sample Buffer) obtained from Bio-Rad and also 1-2µl of 2-Mercaptoethanol,

which is the loading buffer obtained from Sigma Chemical Company, St. Louis, MO, USA. This mixture was boiled for 10 minutes and then added into the wells of Criterion™ TGX™ Precast Gels obtained from Bio-Rad, along with a standard ladder (Precision Plus Protein Western C™ Standards obtained from Bio-Rad. After loading the sample, the proteins were separated by adjusting the volts to 125V and current to 65mA for an hour approximately.

The main purpose of western blotting was to probe the proteins separated on the gel using an antibody specific for one of the proteins and for this purpose, the proteins should be transferred to a thin membrane that allows them to be stabilized for washing, probing and finally allows the antibody to gain an easy access to the proteins. Polyvinylidine Difluoride (PVDF) was used as a blotting membrane in this experiment. For making a gel/PVDF sandwich, the cassette was opened and was dipped into the transfer buffer poured in a tray. After running the gel to a fixed point (to avoid running of the proteins until bottom and lose proteins), it was placed in between two blotting pads which were placed in between the filter papers on both the sides. The PVDF membrane was activated by soaking it in 50% Methanol for 5 min. It was then placed upon the gel such that the proteins upon the gel can be transferred on to the PVDF membrane (No air bubbles should be observed under the PVDF membrane, as that would cause inefficient or no transfer in that area). Another pre-soaked blotting pad was placed upon it, covered with a pre-soaked filter paper and slowly close the sandwich with a knob at the top of it. The whole sandwich was placed in the blotting apparatus such that the membrane was on to the “positive” side. Transfer buffer was added into the apparatus until the diodes were

covered. The transfer was set for overnight maintaining the voltage as 50V and current as 100mA.

Next day the membrane was probed with an antibody. The membrane was blocked to prevent the antibody binding to it via hydrophobic interactions, the membrane was added with a mixture of 3% non-fat dry milk (0.6mg for 20ml of 1X PBS - Tween solution), 20ml of PBS and 0.3% Tween into it. This was added to the PVDF membrane placed in a glass bowl and blocking it for an hour at room temperature with rocking upon the belly dancer/ rocker. After 1 hour it was washed with 1X PBS – Tween for 10 min. The mouse primary antibody, ab3282 Ms mAb to Surfactant Protein B(Mature) obtained from Abcam, diluted at 1:250 was added to 5ml of 1X PBS-Tween for an hour and again washed with 1X PBS-Tween for 10 min. The secondary antibody, ImmunoPure Antibody Goat Anti-Mouse IgG HRP obtained from Pierce (Lot#FJ9240713), was administered to it and placed upon the shaker at least for 30 min. Then washed with 1X PBS – Tween and 1 ml each of substrates, Prod # 1859022 Super Signal West Femto Luminol/Enhancer solution and Prod # 1859023 Super Signal West Femto Peroxide Buffer were added to the PVDF membranes. They were then examined under the ChemDoc MP imaging camera (Bio-Rad, Hercules, CA) for 10 min under Chemi High Sensitivity, Chemi High Resolution and Chemi for Criterion Gel and the images were saved for evaluation. After imaging of the protein by probing surfactant – B antibody, it was stripped with the Restore ^{TM} Western Blot Stripping Buffer Prod # 21059 obtained from Thermo Scientific, Rockford, IL. Then it was washed for some time with the wash buffer, 1X PBS - Tween. It was blocked by using the blocking buffer of 3% non-fat dry milk in 1X PBS- Tween solution for an hour. Then it was washed with the wash buffer and added with 1:500

dilution (20µl of β- actin in 10 ml of wash buffer) of the β- actin primary antibody {Actin (H-300) sc- 10731, Lot# L0612 rabbit polyclonal IgG} obtained from Santa Cruz Biotechnology. It was left to probe for an hour and after washing it with the wash buffer, it was added with the secondary antibody (Prod # 32260 Pierce Goat Anti-Rabbit Poly-HRP) obtained from Thermo Scientific with a dilution of 1: 30,000, i.e. 1µl in 30ml of 1X PBS- Tween solution. It was then washed with wash buffer and 1ml each of the substrates, Prod # 1859022 Super Signal West Femto Luminol/Enhancer solution and Prod # 1859023 Super Signal West Femto Peroxide Buffer were added for examining under the ChemiDOC MP imaging Camera. The molecular weight and density of the protein added with β- actin antibody and Surfactant – B were analyzed for comparison.

Western blotting for leptin receptor protein

The same procedure was followed for the Leptin Receptor Protein too. After blocking the PVDF with blocking buffer 3% Non-Fat Dry Milk in 1%PBS- Tween buffer for an hour, it was washed for 5 minutes with the wash buffer (1%PBS-Tween) and then probed with L9536 Anti-leptin Receptor Antibody produced in Goat (Lot#SLBH9793V) obtained from Sigma, 1:250 dilution in 10ml of blocking buffer for an hour. It was then washed with the wash buffer for 5 minutes and then probed with the Donkey Anti-Goat Polyclonal Secondary Antibody (Cat#AB6885-1) obtained from Abcam. After washing with the wash buffer for 5 minutes, it was added with 1 ml each of substrates, Prod # 1859022 Super Signal West Femto Luminol/Enhancer solution and Prod # 1859023 Super Signal West Femto Peroxide Buffer were added to the PVDF membranes and examined under the ChemDoc MP imaging camera (Bio-Rad, Hercules, CA) for 10 min under Chemi High Sensitivity, Chemi High Resolution and Chemi for Criterion Gel and

the best image was saved for evaluation. After imaging of the protein by probing Anti-Leptin Receptor antibody, it was stripped with the Restore ™ Western Blot Stripping Buffer Prod # 21059 obtained from Thermo Scientific, Rockford, IL. Then it was washed for some time with the wash buffer, 1X PBS - Tween. It was blocked by using the blocking buffer of 3% non-fat dry milk in 1X PBS- Tween solution for an hour. Then it was washed with the wash buffer and added with 1:500 dilution (20µl of β- actin in 10 ml of wash buffer) of the β- actin primary antibody {Actin (H-300) sc- 10731, Lot# L0612 rabbit polyclonal IgG} obtained from Santa Cruz Biotechnology. It was left to probe for an hour and after washing it with the wash buffer, it was added with the secondary antibody (Prod # 32260 Pierce Goat Anti-Rabbit Poly-HRP) obtained from Thermo Scientific with a dilution of 1: 30,000, i.e. 1µl in 30ml of 1X PBS- Tween solution. It was then washed with wash buffer and 1ml each of the substrates, Prod # 1859022 Super Signal West Femto Luminol/Enhancer solution and Prod # 1859023 Super Signal West Femto Peroxide Buffer were added for examining under the ChemiDOC MP imaging Camera. The molecular weight and density of the protein added with β- actin antibody and Anti-Leptin Receptor Antibody were analyzed for comparison.

Protein determination through Bradford assay

The quantification of proteins has been done for the extracted cells treated with the above mentioned drugs before performing western blotting. Plastic test tubes were filled with 800, 799,798, 795, 790, 785 and 780 µl of water. Then added with 1, 2, 5, 10, 15 and 20 µl of 1mg/ml BSA standard to each tube and finally with 200 µl of Bio-Rad Reagent and were vortexed until mixed properly. Then the samples were named as 1, 2, 3, 4, 5, 6, 7, 8 for 24 hour drug treated samples and 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8 for

48 hour drug treated samples. Distilled water (798 µl) was added to each tube, followed by addition of 2 µl of the sample and 200 µl of Bio-Rad Reagent to each tube. All the tubes were vortexed thoroughly and then all of the standard and sample aliquots of 200 µl were added to a 96 well plate for obtaining the results under the Fusion plate reader within an hour.

RT-qPCR:

As the Real Time PCR Analysis was used to determine the expression levels of surfactant - B mRNA in the NCI441 cell line treated with the tocolytics, betamethasone and leptin. The RNA in the samples are quantified and then converted into cDNA by a process called reverse transcription. Then this cDNA is used as a template for exponential augmentation of the given template by using PCR.

Before performing the Real Time PCR Analysis, the RNA Extraction was performed using the **Trizol Method**. The cell culture was performed as described above and after 24 and 48 hours of addition of different drugs, the media was collected into Eppendorf tubes. Trizol reagent (500µl) was added to each in the 12-well plates and incubated for 5 minutes at room temperature. Then this solution turns a bit viscous as the cells lyse, releasing RNA which is then collected into 2ml Eppendorf tubes. Chloroform (200µl) was administered and vortexed until the solution was homogenous. Then it was centrifuged for 15 min at 13,000 rpm; 4^0C. After 15 min the tubes were removed very slowly from the centrifuge so as to avoid mixing of the phases, as we can observe 3 phases, lower red phenol/chloroform phase, a jelly like interphase and colorless upper phase. The colorless upper aqueous phase was transferred into a fresh tube, administered

with 500µl of isopropyl alcohol and incubated for 10 minutes at room temperature. It was then centrifuged for 10 minutes at 13,000rpm; 4^0C and observed a gel like pellet which was formed at the bottom of the tube. The supernatant was slowly decanted and 1ml of 80% ethanol was added to this RNA pellet. It was again centrifuged for 5 minutes at 13,000 rpm; 4^0C, the supernatant was again decanted and the sample was left for drying for more than an hour at room temperature in the sterile hood. It was re-dissolved in 100µl of DEPC RNAse free water and incubated at 57^0C for 10 minutes. It was added with 12µl of reaction buffer, 4µl of DNAse I and was incubated at 37^0C for 1 hour with slight shaking. 100µl of Acid Phenol Chloroform was added to it and placed in ice for 10 minutes. The samples were gently mixed by hand and centrifuged for 5 minutes at 11,300 rpm. Then the upper aqueous phase was transferred into a fresh tube and precipitation of the RNA was performed after addition of 12µl of 3 M sodium acetate, pH 5.5 and 560µl of ice cold 100% ethanol and placing them in -80^0C for overnight. The next day, these samples were centrifuged for 5 minutes at 13,000 rpm, the supernatant was decanted and the sample was dried for more than an hour in a sterile hood at room temperature. The obtained RNA was re-dissolved in 100µl of DEPC RNAse free water and was placed in -80^0C until use.

RNA quantification using Ribogreen reagent

The quantification of RNA in the sample after being extracted by the Trizol method was performed by using the Quant-iT™ RiboGreen kit obtained from Life Technologies. The TE buffer was diluted to 1x from the 20x provided in the kit and all other dilutions were similar to the manual provided along with the kit. As it has been specified that the final volume of experimentation in microplate instead of cuvettes to be

200µl instead of 2µl. So 1x TE was used to dilute both ribogreen reagent and standard RNA provided in the kit. All the standards and samples were made into triplicates in the microplate so that the standard deviation of them could be exactly calculated with only a minimal error and their average has been taken into consideration for the quantification of RNA. The stock standard solution given in the kit was 100mg/ml, which was diluted to 2mg/ml and eventually to 100mg/ml which was considered as a low range assay. This final stock solution was added as 0, 2, 10, 50, 100 µls in triplicates along with 100, 98, 90, 50, 0 µls of TE buffer and all of them with 100 µl of ribogreen reagent at the end to obtain a final volume of 200 µl making those standards as blank, 1, 5, 25 and 50 ng/ml concentrated. In all other wells, 98 µl of TE buffer was administered with 2 µl of the sample in triplicates, finally with 100 µl of ribogreen to make all of them 200 µl. They were then labelled properly and analyzed by using Gen6.1 software in the Synergy – H1 fluorescence microplate reader obtained from Biotek Instruments, Inc (Winooski, Vermont, USA) setting a standard wavelength of excitation~480 nm and emission~520 nm, exported into excel sheet and calculated for a final quantification similar to the Bradford Protein Assay. The whole process has been performed under very dim light and no direct exposure to light to avoid photo bleaching of the working solution. In this assay, the binding of ribogreen to the RNA present in the standard and sample was very critical for providing accurate values. Quantification of RNA in the samples was very important for estimating the RNA content in the samples, for an accurate dilution factor for maintaining a minimal value equal for all the samples in order to get good peaks in the RT-PCR.

Reverse transcription for obtaining cDNA

The obtained concentrations of all the samples of 24 hour and 48 hour have been equalized for each set to a make it 100ng to 1000ng concentration depending upon the lowest value in the set. Then the RNA of each sample is diluted to a final concentration of 30µl and from that only 7.5µl is used for the reverse transcription process. The supermix iScript Reverse Transcription Supermix for RT-qPCR Cat#170-8841 obtained from Bio-Rad has been diluted with nuclease free water as per the protocol for example 413µl of nuclease free water was added to 150µl of the supermix to dilute it before the addition of RNA to it. Then 22.5µl of this mixture was administered to 7.5µl of the RNA sample to each well for obtaining a final volume of 30µl per each well, as we need 5µl each of the c DNA template for obtaining the forward template through PCR reaction finally. As we have designed every sample to be in triplicates, we need 30 µl each well of the c DNA for obtaining 5µl of the template for 3 wells for Surfactant – B and 3 wells for β – Actin of the final PCR reaction plate. Bio-Rad icycler iQ PCR plates, 96 well was used and the plate was sealed with CFX96 Optical Quality Sealing tape obtained from Bio-Rad and placed in the CFX96 Bio-Rad Real Time PCR Machine for undergoing the process of reverse transcription. The complete mix has been incubated in thermal cycler by using the protocol in which the step was to undergo priming at a temperature of 25°C for 5 minutes, followed by Reverse Transcription (RT) at a temperature of 42°C for 30 minutes and finally inactivating the RT at a temperature of 85°C for 5 minutes which is usually followed cooling the plate for 30 minutes at a temperature of 4°C.

Real Time PCR analysis

After the conversion of the RNA to cDNA, we had to analyze the expression levels of the cells with no drug and the cells added with various drugs. For this, we

prepared two Mastermixes one each specific for Surfactant–B containing surfactant–B primers and β–actin here which was used as the housekeeper gene for these set of experiments having β–actin primers. So the Mastermix which consists of SsoAdvanced Universal SYBR Green Supermix Cat# 172-5271 obtained from Bio-Rad which was administered to the mastermix as 10µl per well, 1µl each of the forward and reverse primers of specific protein (either surfactant – B or β – Actin) and finally made to 15µl of the mastermix per each well by the addition of 3µl of water per well. So, this mastermix is tapped at the bottom of the microfuge tube for thorough mixing. After spinning the tube, 15µl of the surfactant – B mastermix is added to the first row and β – Actin mastermix to the next row and so on arranging both of them alternatively for an easy quantification. Then 5µl of the previously obtained template is added to each of the triplicates of surfactant – B and β – Actin by pipetting up and down according to a pre-designed arrangement of the plate setup.

This is the plate setup used for the 24 hour drug sample for performing RT- qPCR analysis in which 1.1, 1.2, 1.3 were controls; 2.1, 2.2, 2.3 were treated with betamethasone; 3.1, 3.2, 3.3 were treated with progesterone; 4.1, 4.2, 4.3 were combination of betamethasone and progesterone; 5.2, 5.3 were terbutaline alone; 6.1, 6.2, 6.3 were combination of terbutaline and betamethasone; 7.1, 7.2, 7.3 were leptin alone; 8.1, 8.3 were combination of leptin and betamethasone. NTC were no template controls for both surfactant–B and β – Actin.

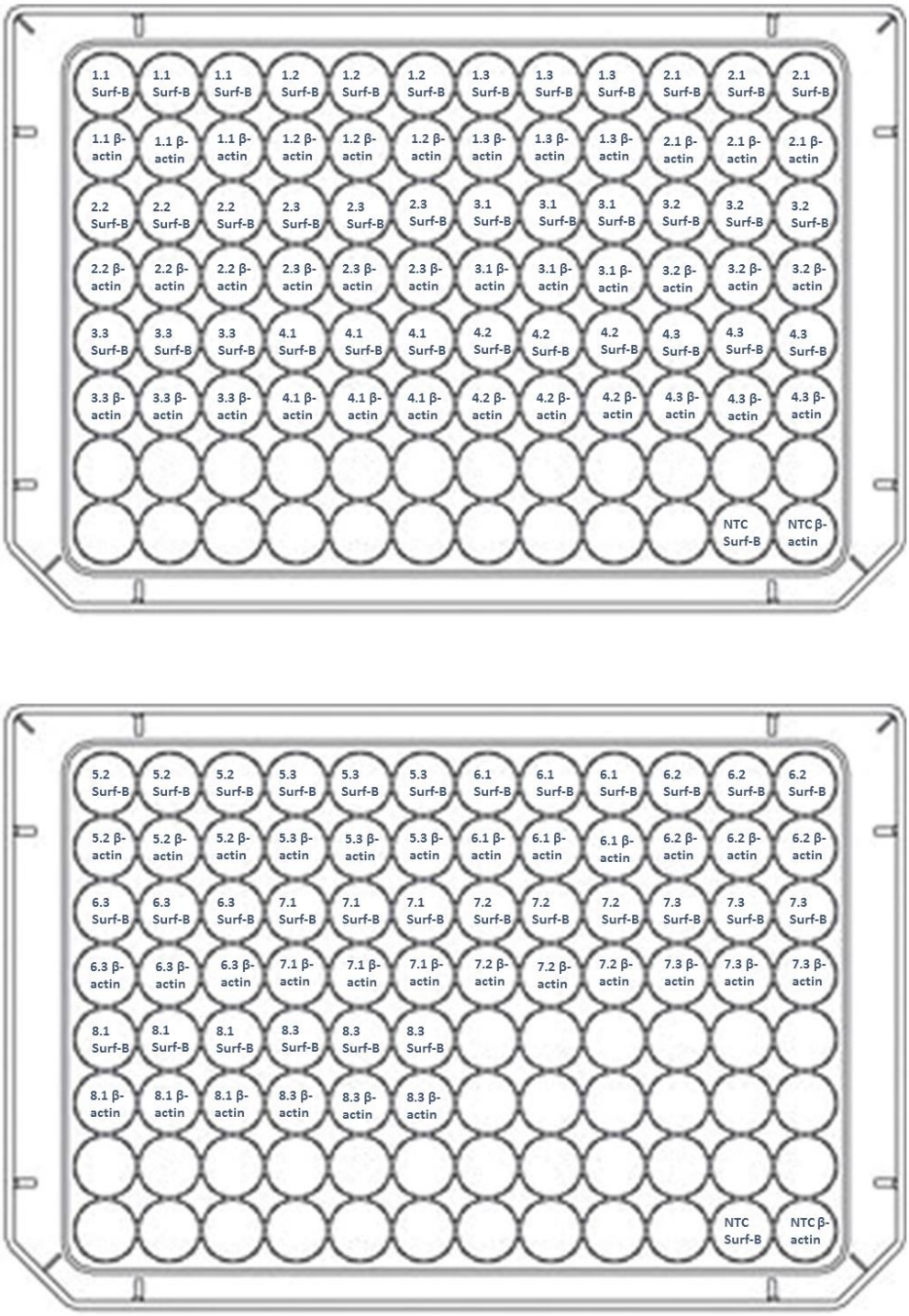


Figure 5: RT- PCR Plate schemes for all the PCR analysis experiments. The plate scheme is as follows: 1 - control, 2 - betamethasone, 3 – progesterone, 4 - progesterone in combination with betamethasone, 5 – terbutaline, 6 – terbutaline in combination with betamethasone, 7 – leptin and 8 – leptin in combination with betamethasone. All the above treatment groups are analyzed in triplicates.

The plate was incubated in the thermal cycler by following the previously designed protocol after performing many trials. First the gradient temperature has been tested from 40°C to 52°C for 2 – 3 times from which there were good expression levels and Cq values seen for the 48°C annealing temperature. We have even tried with 35 cycles to 50 cycles for obtaining a good quantification data. The final protocol followed during the actual experiment for all the samples in triplicates was maintained at 95°C for 30 seconds for the polymerase activation and DNA denaturation, then the 3 step cyclic process for 50 cycles in which the first step is denaturation at 95°C for 15 seconds followed by 48°C of annealing for 30seconds and 60°C for 30 seconds for extension and plate read. After this process is being repeated for 50 cycles, 65°C - 95°C 0.5°C increment 2-5sec per step for obtaining the melt curve. Finally it is kept on hold at 4°C until removed. After obtaining the data of the Cq values and the melt curves, graphs have been plotted according to the data.

Results

TLC analysis of lipids from lung cells in response to tocolytics:

Tables 2 and Table 3 show the mean Retention Factor (R_f) values of 24 hour drug treated and 48 hour drug treated media analyzed for phospholipids present in it through Thin Layer Chromatography technique by spotting the sample and standard lecithin or sphingomyelin upon HPTLC plates. The retention factor is calculated for all the 4 bands appeared upon the HPTLC plates after analyzing them at 625nm for all the samples (triplicates) and also the standard spotted upon each plate. These values allow proper comparison of the standard and the four bands of the sample for a clear differentiation of the phospholipids, lecithin and sphingomyelin. NA represents not applicable as only 1 standard has been spotted on the plate. The average sample R_f values have been shown in these tables. From this R_f analysis, we determined that the top band aligned most closely with lecithin and the third band aligned more closely with sphingomyelin. Unfortunately, the results were not an exact match owing to different Rfs for different plates. Mass spectrometry will provide a more definitive identity in the future.

Table 2: Retention Factor (R_f) values for 24 hour TLC						
Treatment	**Band 1**	**Band 2**	**Band 3**	**Band 4**	**STD L**	**STD S**
Control	0.29	0.35	0.44	0.51	0.36	NA
Betamethasone	0.38	0.41	0.50	0.58	NA	0.46
Progesterone	0.35	0.40	0.49	0.56	0.35	NA
Betamethasone+ Progesterone	0.33	0.37	0.48	0.54	0.32	NA
Terbutaline	0.27	0.31	0.40	0.47	NA	0.38
Betamethasone+ Terbutaline	0.29	0.32	0.41	0.46	0.30	NA
Leptin	0.26	0.29	0.38	0.44	NA	0.36
Betamethasone+ Leptin	0.20	0.25	0.41	0.46	0.17	NA

Table 3: Retention Factor (R_f) values for 48 hour TLC						
Treatment	**Band 1**	**Band 2**	**Band 3**	**Band 4**	**STD L**	**STD S**
Control	0.23	0.27	0.36	0.42	0.20	0.34
Betamethasone	0.22	0.26	0.35	0.41	0.21	NA
Progesterone	0.22	0.26	0.35	0.41	NA	0.34
Betamethasone+ Progesterone	0.36	0.40	0.48	0.55	NA	0.34
Terbutaline	0.30	0.36	0.46	0.54	0.27	NA
Betamethasone+ Terbutaline	0.27	0.34	0.44	0.53	NA	0.42
Leptin	0.28	0.33	0.41	0.50	0.29	NA
Betamethasone+ Leptin	0.09	0.17	0.31	0.49	0.09	NA

To determine the effect of betamethasone and the tocolytics on lipid production and secretion from lung cells, we performed TLC analysis of the lipids extracted from cells treated with these drugs. The tocolytics and their concentrations used in this study are shown in Table 1. The extracted lipids were separated on the HPTLC plates in an organic solvent system along with the standards for sphingomyelin and lecithin (phosphatidylcholine). Densitometric analysis of the four bands observed with each lipid spot in all lanes was determined and secretion estimated as the optical density relative to background. Secretion of the lipids from control cells was minimal at 24 hours (Figure 6). Exposure to betamethasone significantly increased lipid secretion (Increase observed in all the bands). Progesterone alone or in combination with betamethasone significantly increased secretion when compared to betamethasone alone (Figure 7; $p<0.05$). Terbutaline showed a significant increase over control but the addition of betamethasone did not cause further increase. Leptin alone provoked 10 fold increase compared to controls ($p<0.05$), but was much lower in the presence of betamethasone, though still about a 4 fold increase over controls ($p<0.05$).

After treatment with the drugs for 48 hour, betamethasone was not found to cause a significant increase in lipid secretion (Figure 8). There was no significant increase in lipid secretion in response to progesterone or progesterone in combination with betamethasone (Figure 9). Lipid secretion was increased at 48 hours compared to control with terbutaline and addition of betamethasone decreased secretion to control levels. Leptin appeared to have no significant effect on bands 1 and 2. In contrast band 3 was decreased and band 4 appeared not to be affected (Figure 9).

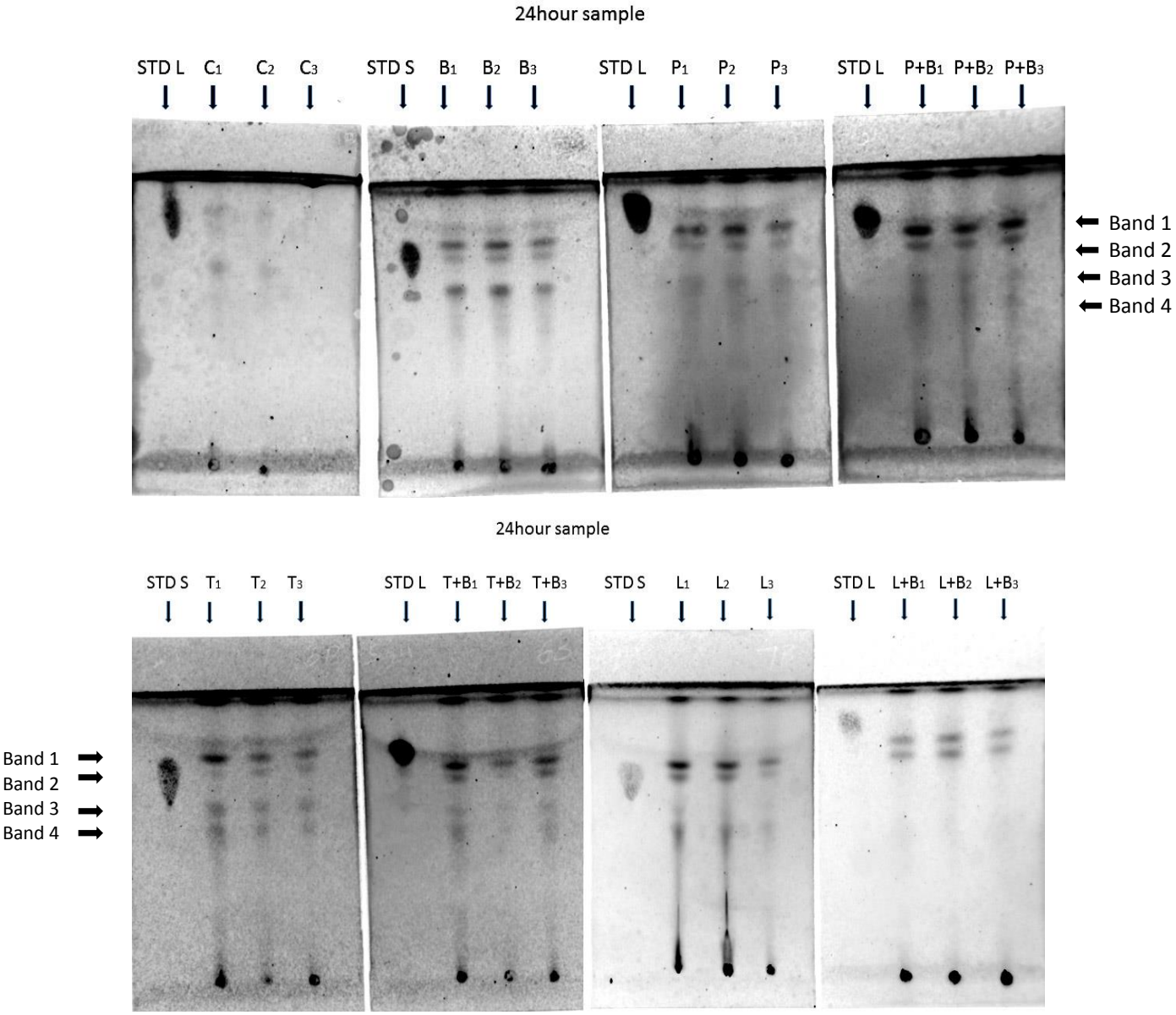


Figure 6: HPTLC analysis of Secreted Lipids from Lung Cells. Both of them are *24 hour* drug added treatment groups. Lipids were extracted from the media, separated by HPTLC plates, and fluorescent images captured at 465 nm. The samples were loaded (4 µl/sample) and run until 1.5 cm from the top of the plate. Each plate represents three independent samples from experiments. Standard and samples have been prepared at same concentrations, however evaporation in the mixture may have resulted in different final concentrations. In the figure, C - control, B - betamethasone, P - progesterone, T - terbutaline, L - leptin.

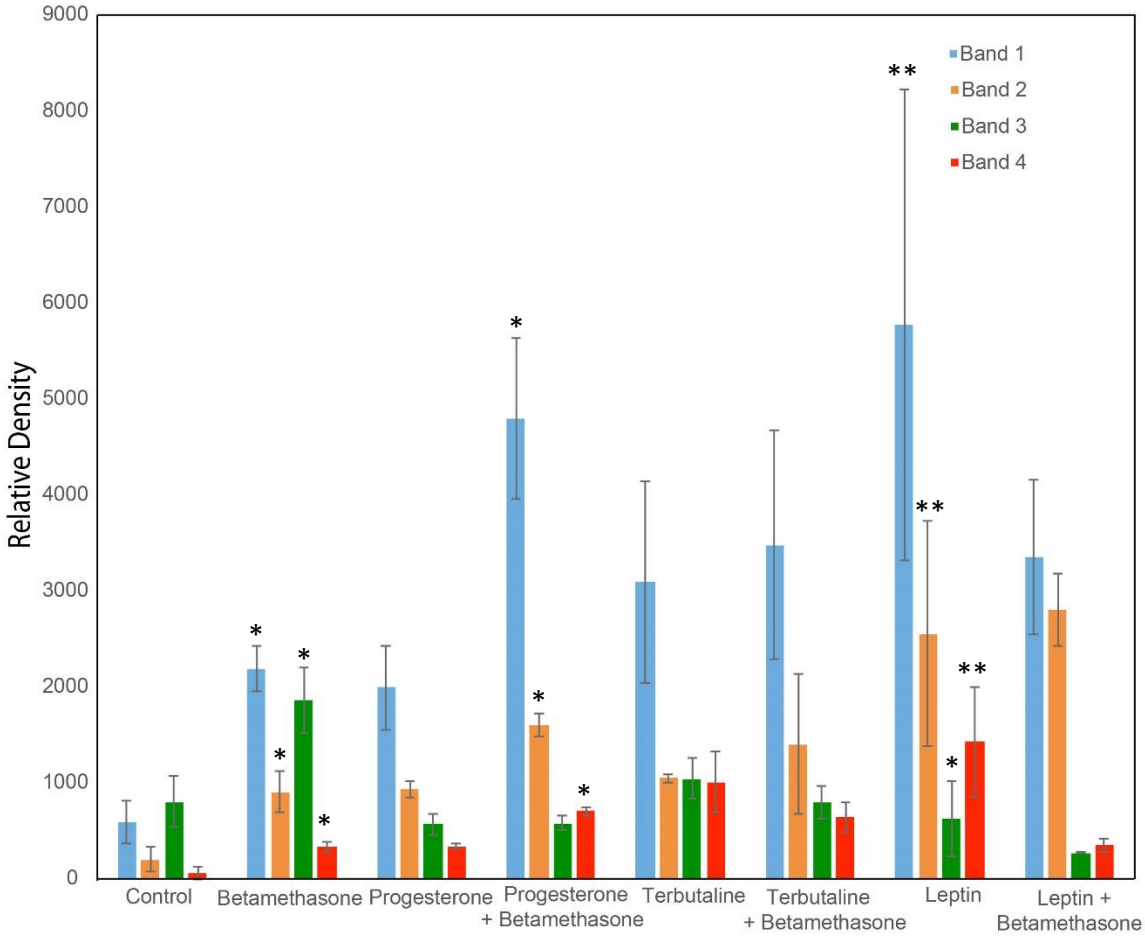


Figure 7: This graph represents the densitometric calculation of the bands of different treatment groups obtained relative to the background through HPTLC's for 24 hour drug addition to those cells**.** The results represent the mean ± SEM for 3 independent wells of cells treated individually. (* implies a significant increase in all the four bands combined together compared to the control, ** implies a significant increase when compared to *)

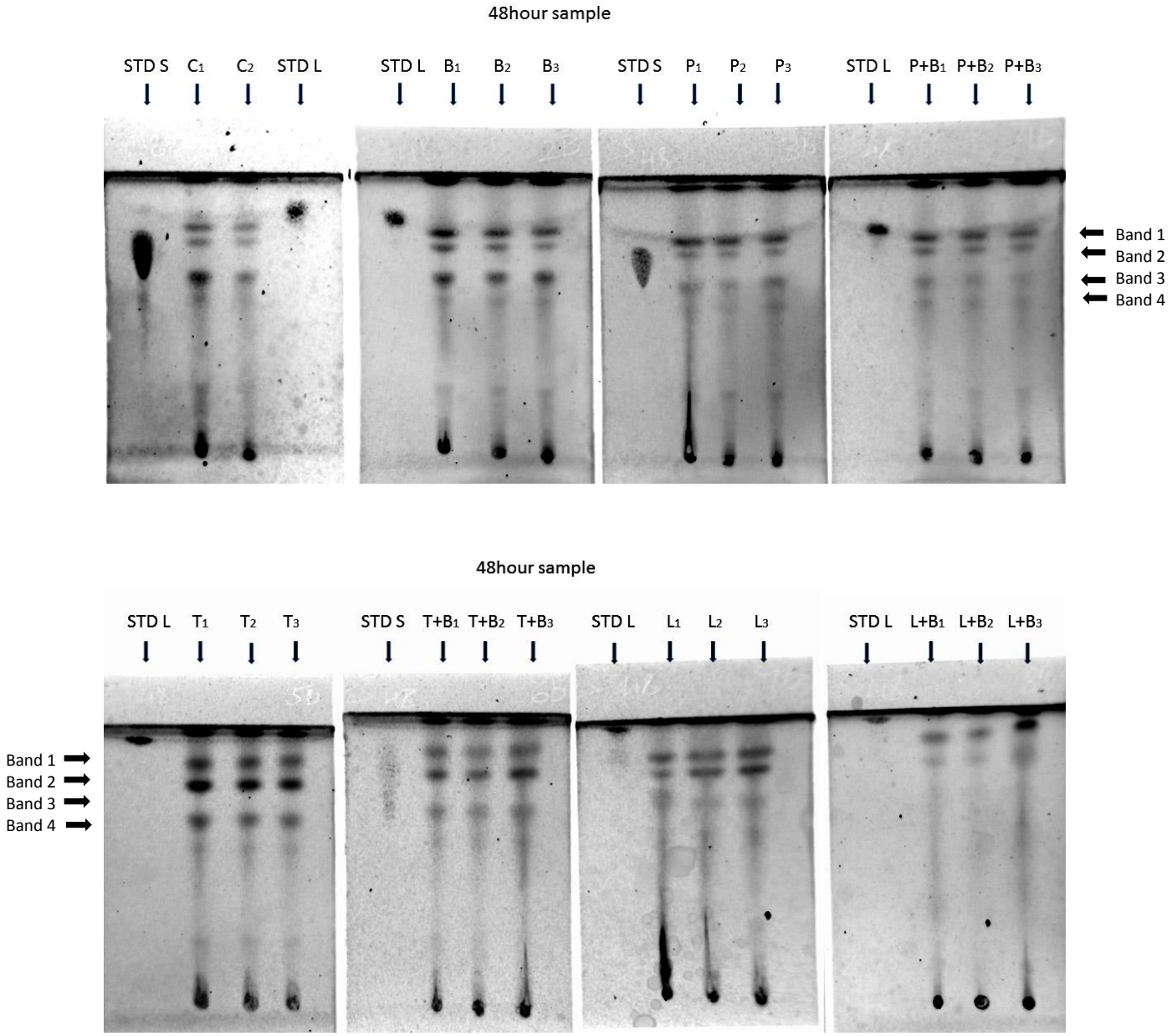


Figure 8: HPTLC analysis of Secreted Lipids from Lung Cells, 48 hours after treatment. Lipids were extracted from the media, separated by HPTLC plates, and fluorescent images captured at 465 nm (The samples were loaded (4 □l/sample) and run until 1.5 cm from the top of the plate. Each plate represents three independent samples from experiments.

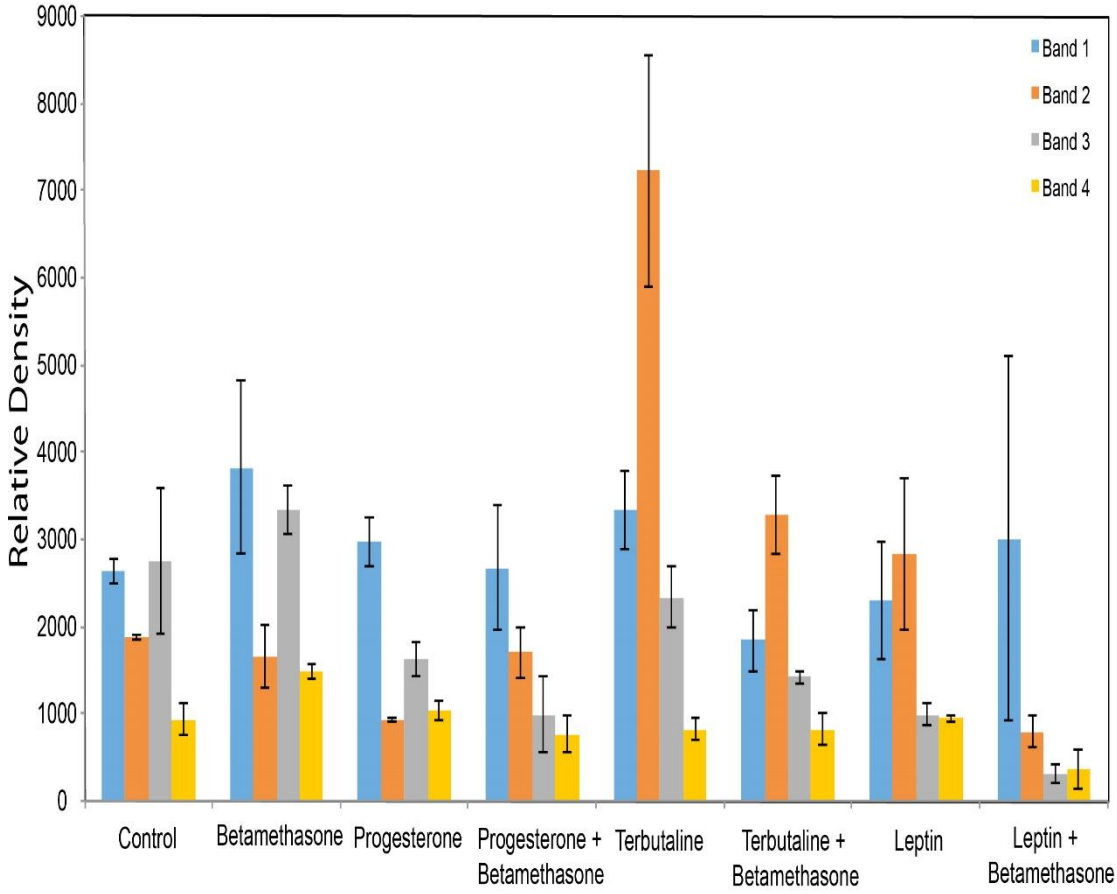


Figure 9: This data represents the densitometric calculation of the bands of different treatment groups obtained relative to the background through HPTLC's for 48 hour drug addition to those cells.

Immunocytochemistry

Having observed a positive effect of the betamethasone, tocolytics and leptin on lipid production and secretion. We wanted to determine if the above mentioned drugs have a similar effect on intracellular surfactant – B secretion and production. To determine the effect of each of the drug treatments on surfactant in the cells, we used immunostaining with an antibody to surfactant-B protein. The cells which were administered with the drugs for either 24 hours or 48 hours were probed with an antibody

for surfactant along with DAPI reagent for staining the nuclei. Images were captured on the fluorescent microscope. At 24 hours, it appeared that betamethasone-treated cells showed an apparent increase in fluorescence signal relative to control cells (Figure 10). At 24 hours, it appeared that betamethasone in combination with progesterone gave a stronger response. On the contrary, terbutaline and leptin both showed increased brightness without betamethasone, which appeared to diminish in the presence of betamethasone. In contrast, the images of 48-hour drug addition gave slightly different results in that the surfactant content was marginally increased (Figure 11). We performed the immunocytochemistry of these cells three times to ensure that the results were reproducible. Altogether, these results suggest that betamethasone with progesterone have a positive influence on surfactant-B production.

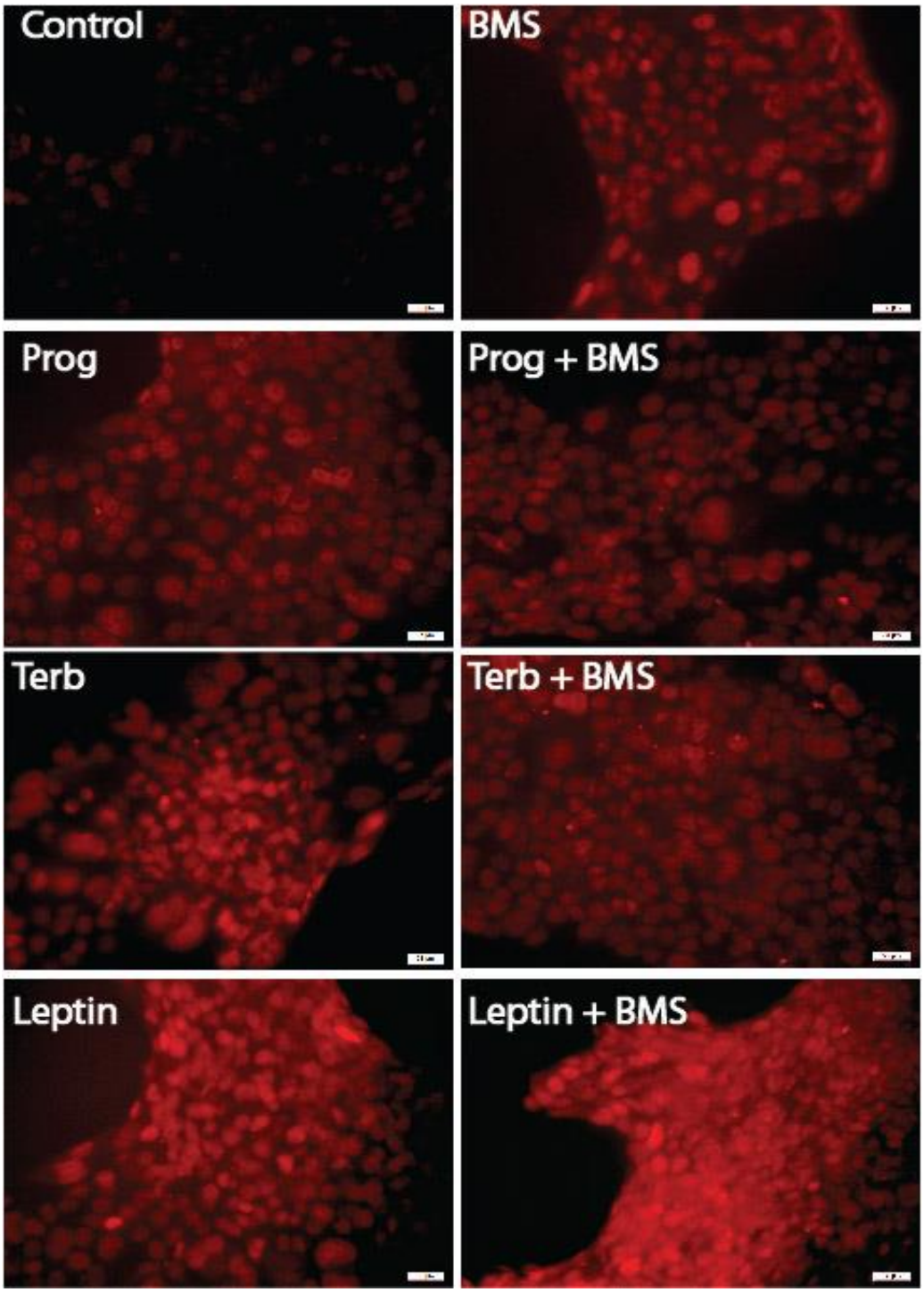


Figure 10: The above figure shows the microscopic view of the fluorescent light upon these cells of H441 adenocarcinoma cells which shows an increase in the brightness or intensity of the lipids after 24 hours of various drug addition to each of them when compared to the control and the positive control, betamethasone (BMS).

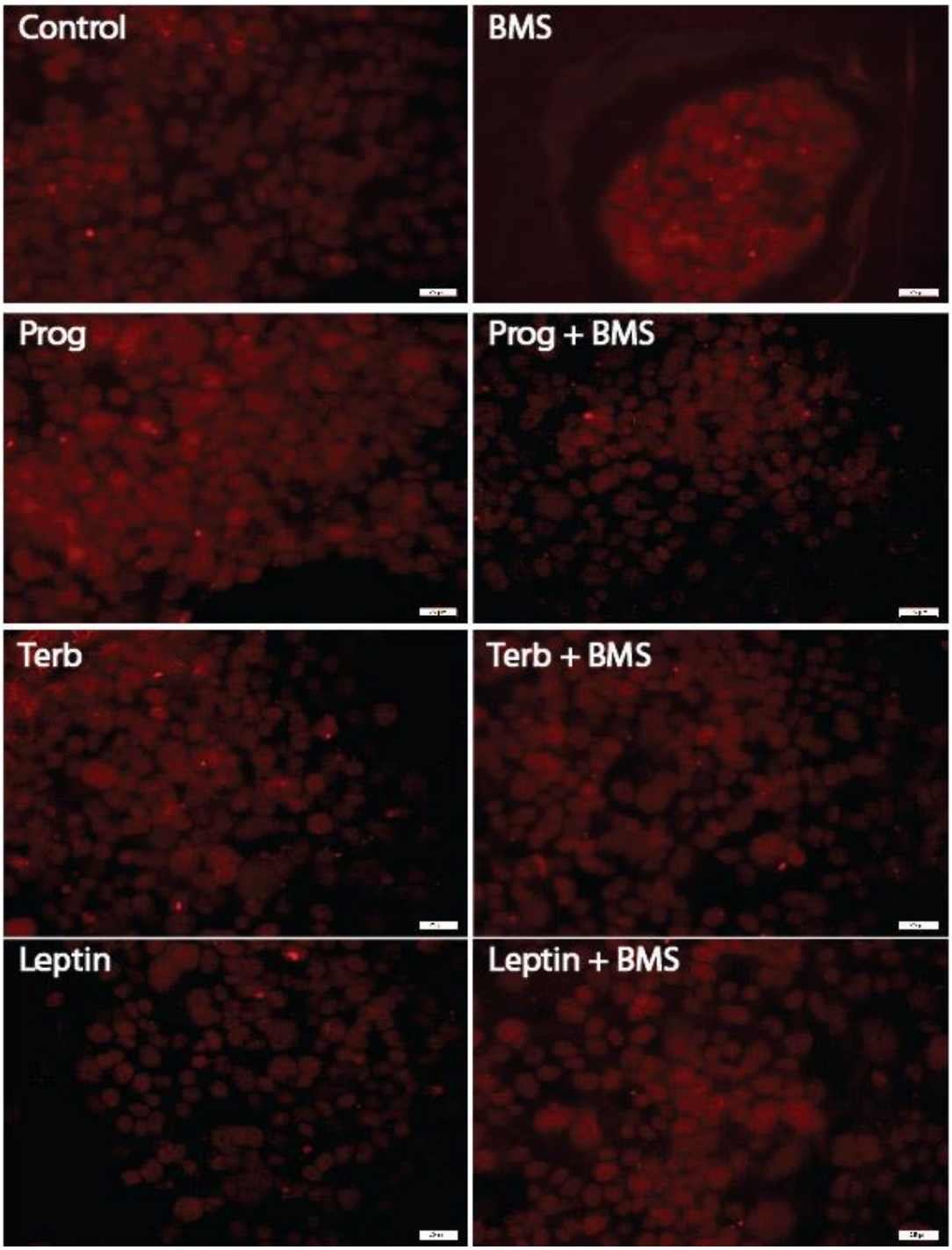


Figure 11: The above figure shows the microscopic view of the fluorescent light upon these cells of H441 adenocarcinoma cells which shows an increase in the brightness or intensity of the lipids after 48 hour of various drug addition to each of them when compared to the control and the positive control, betamethasone (BMS).

Western blotting

Immunocytochemistry is a visual observation type of analysis and can be very subjective depending upon the eyes of the observer, difference in production of protein and lipids among different sets of experiments, number of cells and other factors. Therefore, we used western blotting to determine the effects of the above mentioned drugs on SP-B expression on NCI-H441 cells. Western blotting was completed on the protein in the cells and media and then analyzed densitometrically through image lab software. 24 hour treated cell extracts have shown a profound band for SP-B at ~38 kDa and a doublet band for β-actin at ~42 kDa (Figure 12A). This might strongly be the difference between the protein content (baseline) between different treatment groups in different sets of experiments, which may have caused a bigger standard error, more variability between treatment groups, change in culture conditions and all other uncontrollable factors may have contributed for not showing any significant difference between any of the treatment groups, which can be evident through the individual graphs of different sets measured quantitatively.

At 48 hours, there was a less distinguished band for control and betamethasone treated SP-B, showing a reduction in SP-B expression and also β-actin when compared to the 24 hour control and betamethasone-treated cells (Figure 12B). When the density of each band for each of the treatment groups was analyzed, there appeared to be no significant difference in surfactant-B production at 24 and 48 hours (Figures 13 & 14). The western blot results appear to suggest that SP-B is not significantly affected with any of the drugs used in this study, due to many factors affecting its actual result.

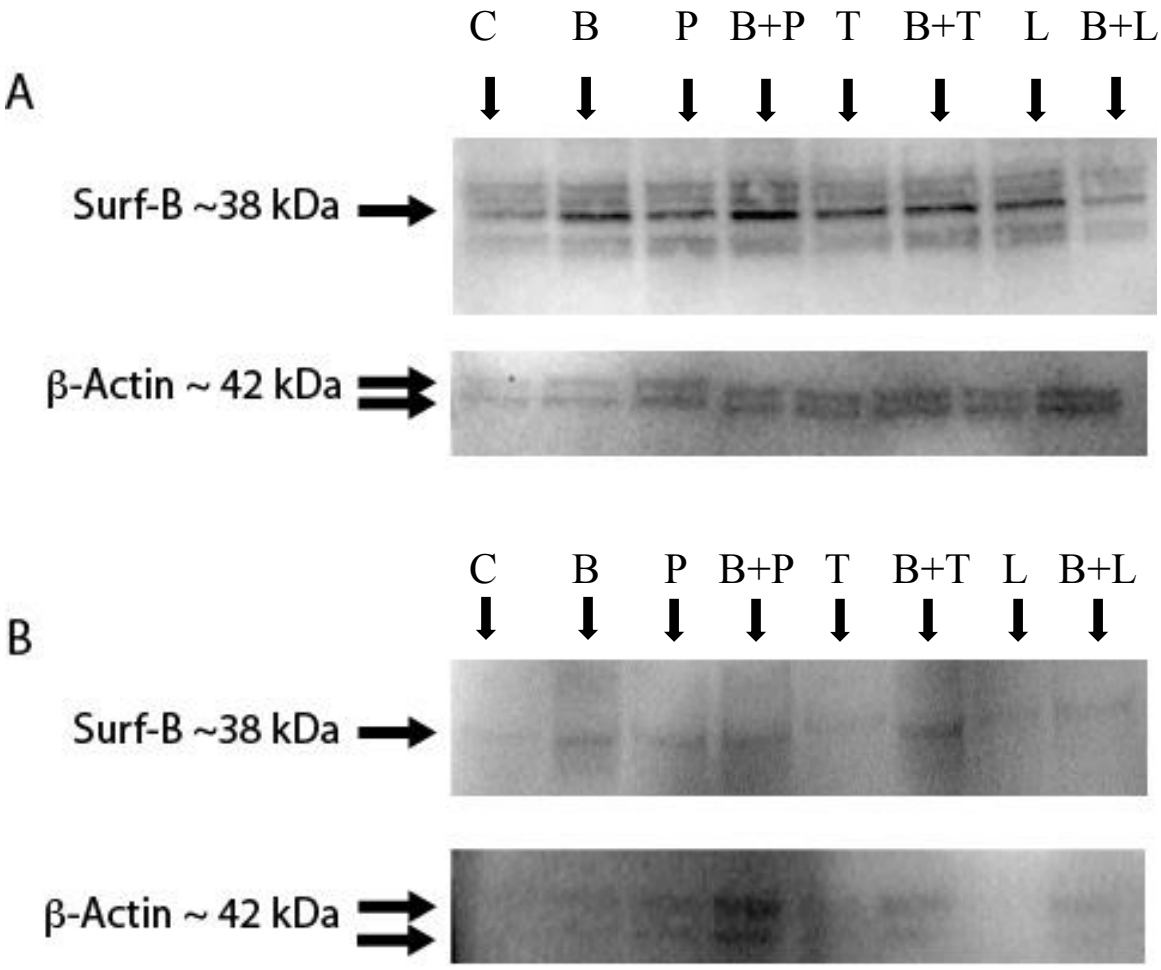


Figure 12: Western blot analysis of surfactant–B and actin in extracts of cells treated with tocolytic agents at (A) 24 hours of treatment with all those drugs for surfactant-B protein found at~38 kDa and β-actin protein at~42 kDa (B) 48 hours of drug treatment for surfactant-B protein and β-actin protein .

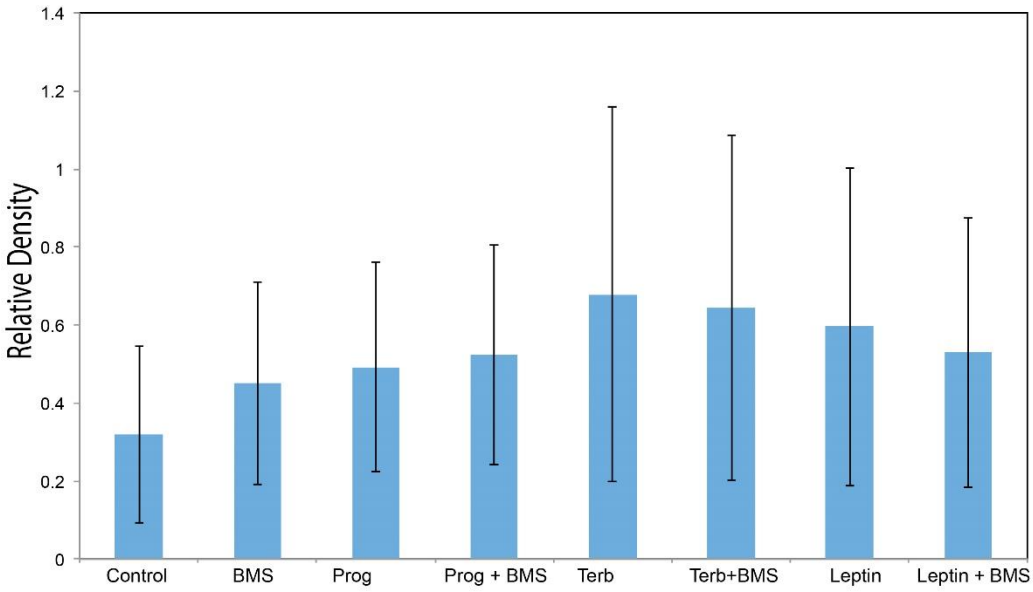


Figure 13: Densitometric analysis of the surfactant bands from 24 hour treatment groups. Surfactant density was normalized to b-actin for statistical analysis. The results represent the mean ± SEM from 3 different determinations for each treatment group or control.

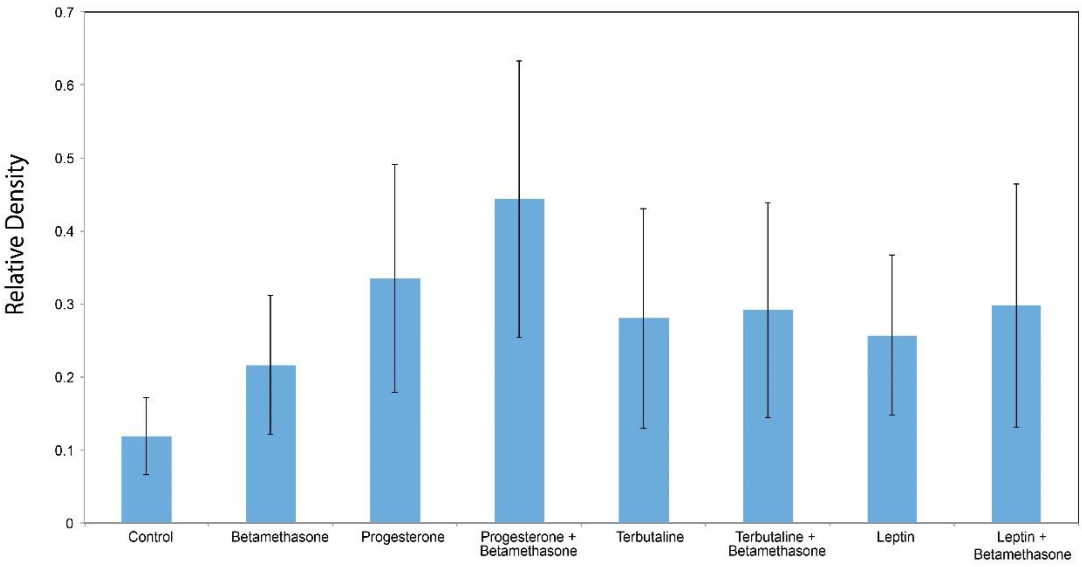


Figure 14: Densitometric analysis of the surfactant bands from 48 hour treatment groups. Surfactant density was normalized to b-actin for statistical analysis. The results represent the mean ± SEM from 3 different determinations for each treatment group or control.

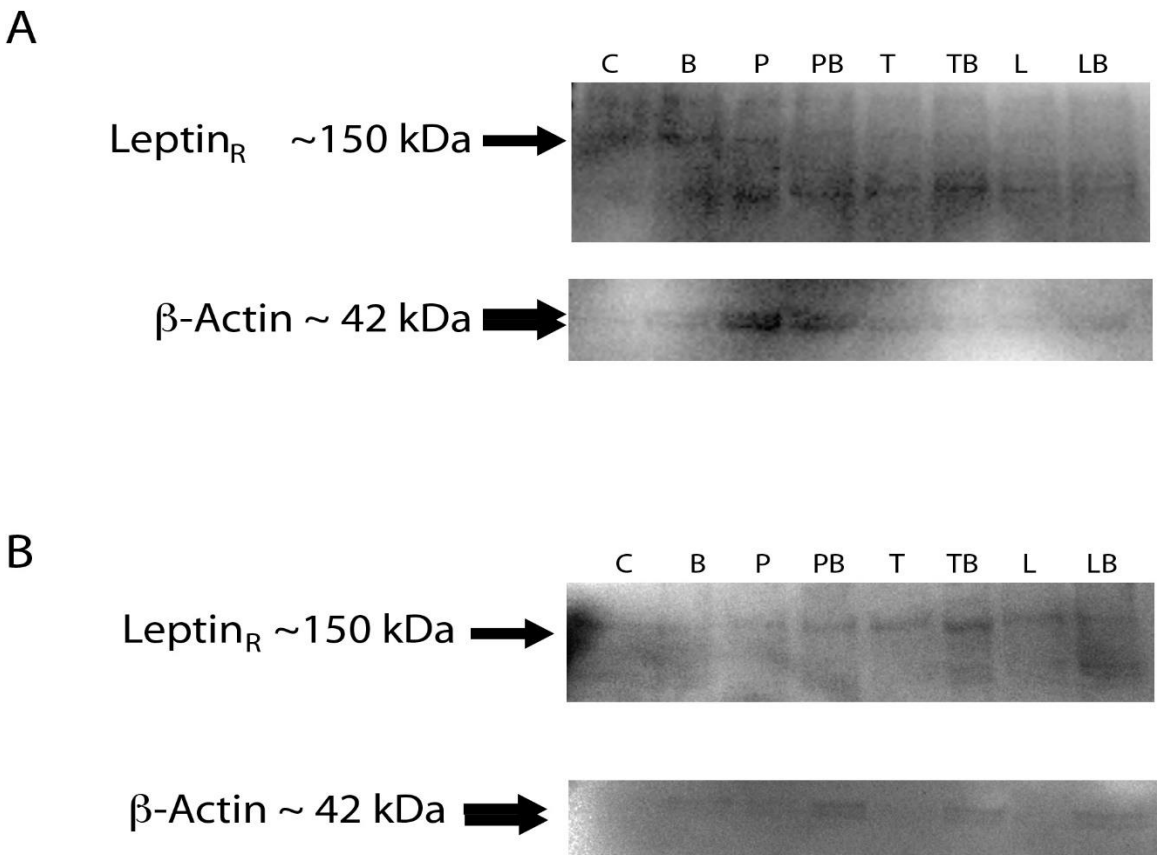
Leptin receptor protein

The first question to be answered about leptin receptors was whether leptin receptor protein was present in the NCI441 cells (mature cells) used in this project. Western blot analysis of the cell lysates showed a faint but discernible band at 150 kDa as predicted for the leptin receptor (Figure 15 A&B). Although we cannot initiate the receptor protein abundance in-vitro, we have tried to see if there was any difference in the receptor protein content after addition of these drugs, as there may be a modification in the protein due to the mRNA changes and other expressions of the cells in presence of these drugs.

After 24 hours of drug treatment, there appeared to be a slight increase in leptin receptor expression in response to betamethasone (Figure 15). In the other treatment groups it appeared as though the 150 kDa band was shifted to a smaller size,~100 kDa and the other as~75 kDa. The beta actin doublet was also present. At 24 hours, the bands were faint and densitometric analysis suggested that there may have been a drug effect, but there was no significant difference due to high variability in different blots.

For 48 hours of drug treated cells, the results were even less pronounced as the bands were barely visible and have made the densitometric analysis very difficult. But by observing the western blots of all the sets of 48 hour drug treated cells have shown either a degradation of protein or the cleavage of the protein. This is because of the appearance of bands at three different molecular weights as mentioned above. This has made even difficult for densitometric analysis, as these degradations were not observed with all the treatment groups repetitively with the same group in all the sets of experiments. Hence

made this a big problem for their densitometric analysis for considering only one prominent band and this led for a greater statistical error for their comparison with the control, though observed by the naked eye. Overall, the results made it difficult to determine anything except that the leptin receptor protein appeared to be present in these mature cell line and proved that they are not only confined to the fetal cell line.



F**igure 15.** Representative images for the leptin receptor protein and beta-actin protein obtained through western blotting. (A) 24 hour treatment with the tocyolytics for leptin receptor protein observed at~150 kDa and cleavage or degradation of protein at~100 kDa and β-actin protein at~42 kDa (B) represents 48 hour treatment with above mentioned drugs for leptin receptor protein observed at~150 kDa and β-actin protein at~42 kDa.

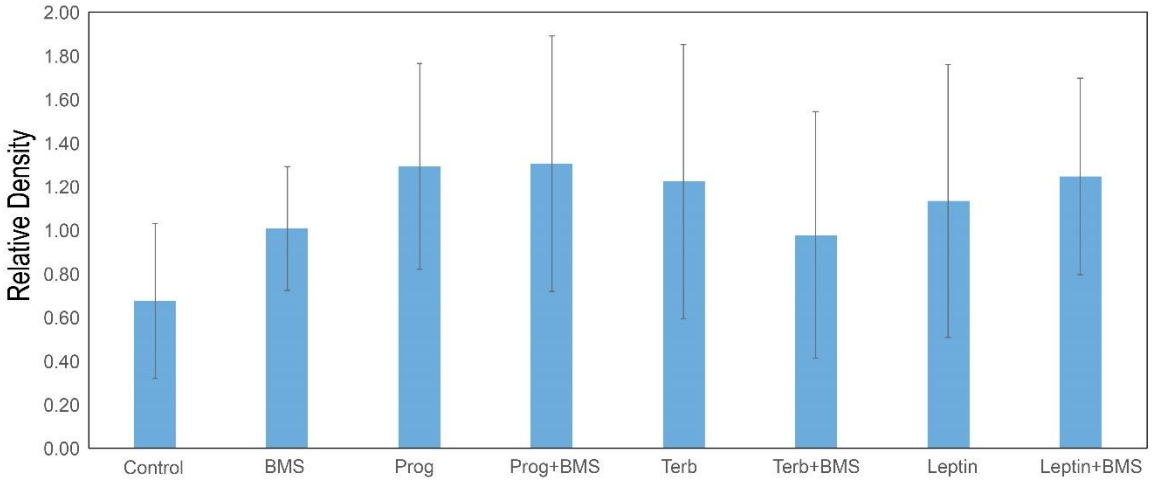


Figure 16: Densitometric analysis of the leptin bands from 24 hour treatment groups. Leptin density from the western blot was normalized to b-actin for statistical analysis. The results represent the mean ± SEM from 3 different determinations for each treatment group or control

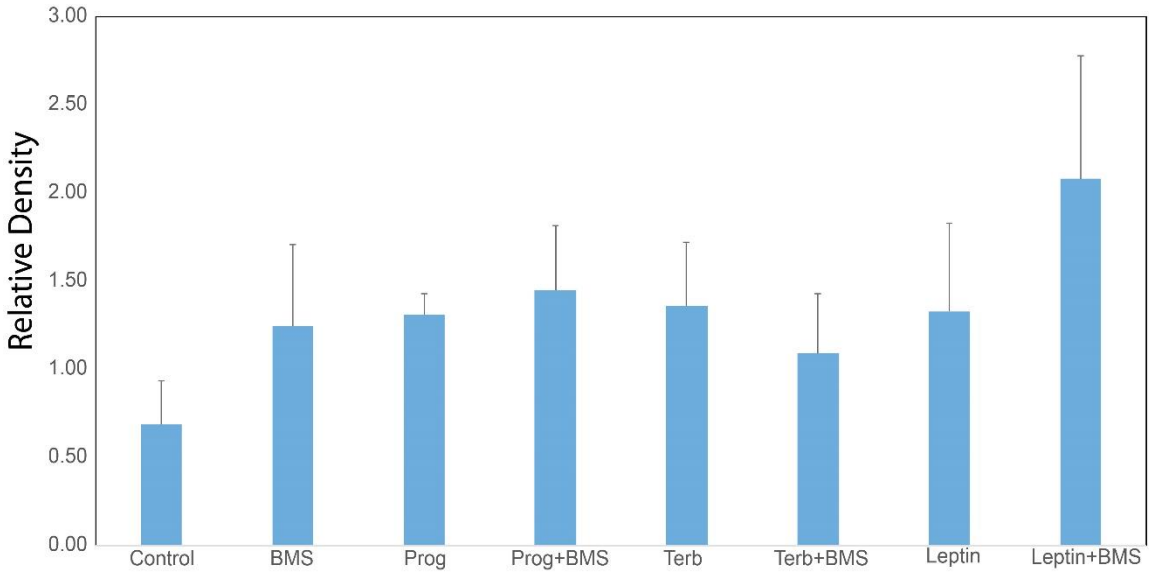


Figure 17: Densitometric analysis of the leptin bands from 48 hour treatment groups. Leptin density was normalized to b-actin for statistical analysis. The results represent the mean ± SEM from 3 different determinations for each treatment group or control.

Real Time - PCR Analysis

Next we analyzed the SP-B mRNA expression. At 24 hours, the RT-PCR showed curves for most of the wells from surfactant-B and β-actin primers. Although a high amount of template was used (990ng) for all sets of experiments of 24 hour drug treated cells with PCR, the curves which are obtained during the quantification of the template by using a Bio-Rad's software for PCR analysis has shown a shift of these curves to the right, suggesting the presence of low amount of mRNA in the template. The curves of actin were similar to the surfactant-B suggesting that the RNA may have been degraded.

At 48 hours, the amount of mRNA that could be used for a template was much lower resulting in the shift of those curves further to the right. This shift pushed the PCR to give curves at or after 50 cycles, preventing us from using the data for ΔΔCT analysis. Hence we can say that the results of 48 hours of drug addition were not significant as determined by statistics with a $p<0.05$ and cannot be considered for final evaluation.

The results from the drug treatment at 24 hours show significant increase in surfactant-B mRNA with the addition of betamethasone versus control, progesterone, and leptin respectively. Progesterone in combination with betamethasone has shown a significant increase when compared to progesterone alone. Leptin with betamethasone has also shown a significant decrease in surfactant-B mRNA when compared to betamethasone alone. These results suggest that Leptin Receptor mRNA is expressed in the mature lung cell line and that it is responsive to betamethasone and other tocolytic treatments.

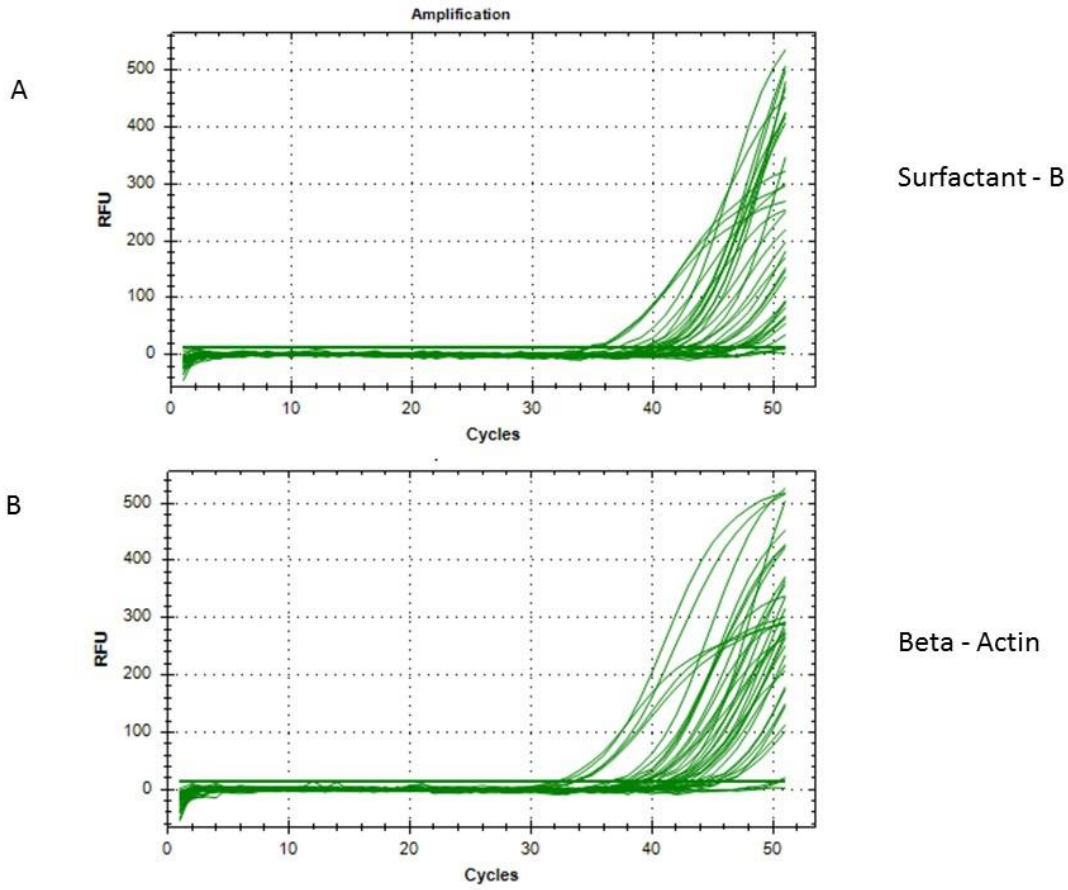


Figure 18: Images obtained through Real time PCR analysis for quantification of expression levels and mRNA secretion of surfactant-B by addition of 24 hours of Control, Betamethasone, Progesterone and Progesterone in combination with betamethasone, the positive control (samples 1.1 -4.3 as shown in the plate setup, analyzed in triplicates) A. Shows curves obtained with Surfactant-B primers and B. Shows curves obtained with Beta-Actin primers.

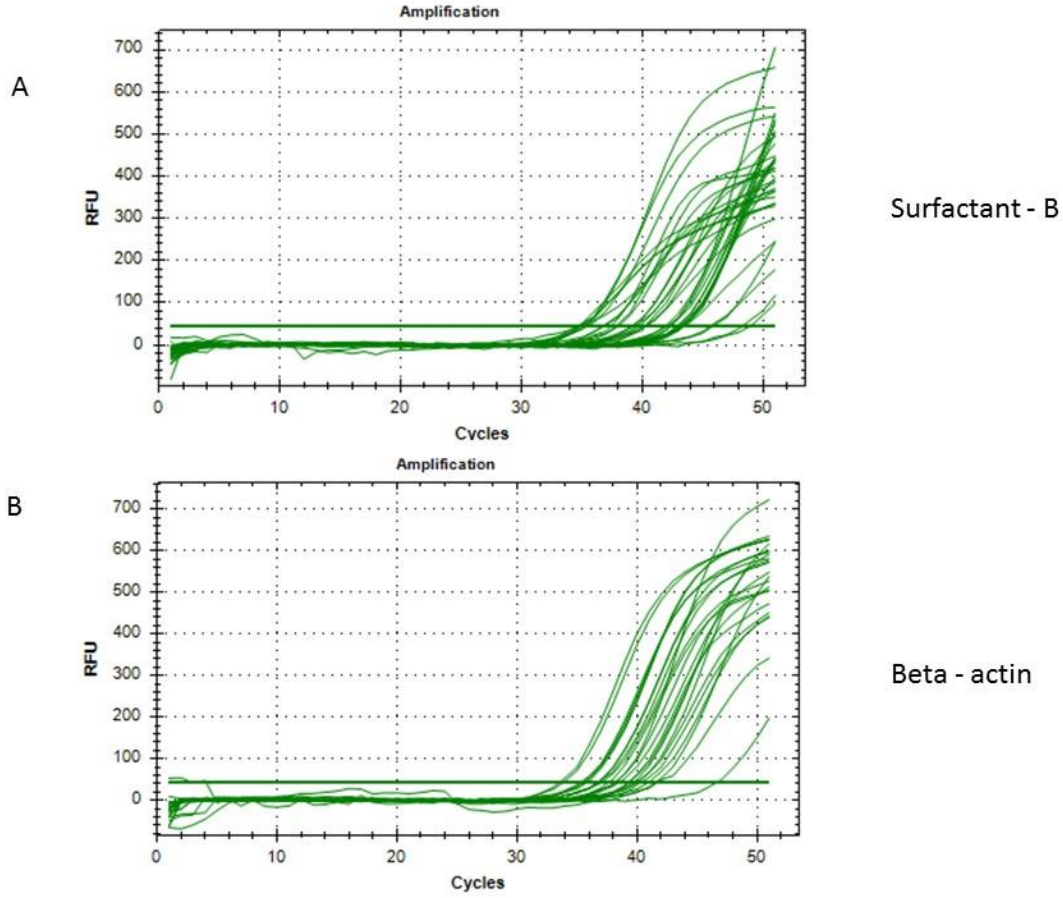


Figure 19: Images obtained through Real time PCR analysis for quantification of expression levels and mRNA secretion of surfactant-B by addition of 24 hours of terbutaline, terbutaline in combination with betamethasone, leptin alone and in combination with betamethasone (Samples 5.2 -8.3 as shown in the plate setup, analyzed in triplicates) A. Shows curves obtained with Surfactant-B primers and B. Shows curves obtained with Beta-Actin primers.

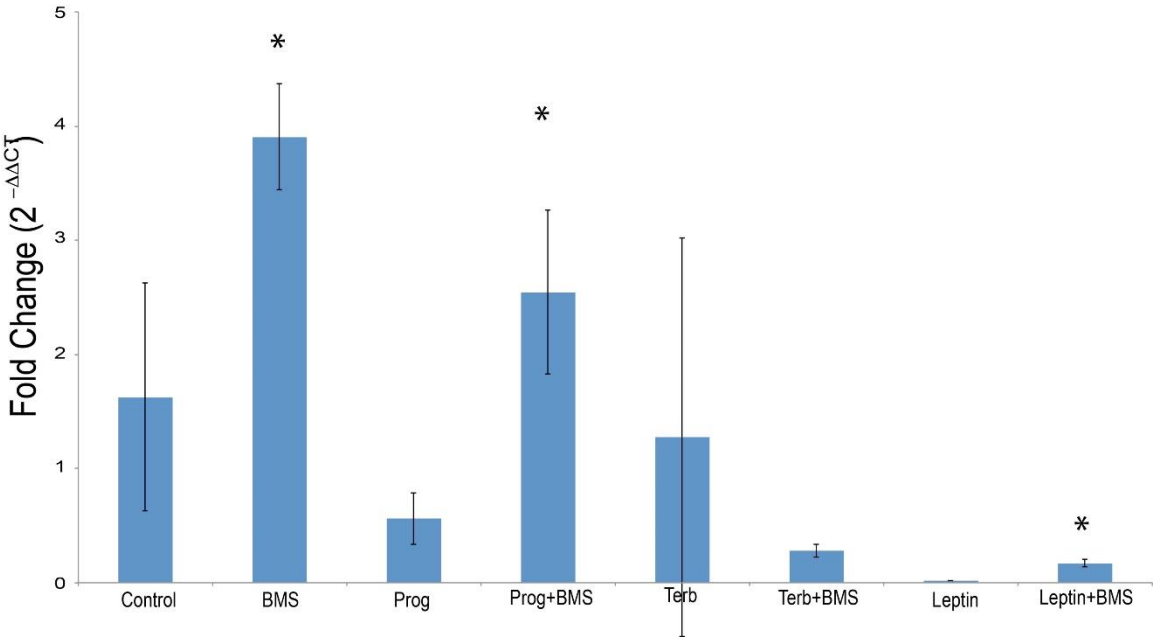


Figure 20: ΔΔCT analysis of qRT-PCR results for lung cells treated with different tocolytics. Here the Y-axis represents the relative change according to the 2^-DDCT results and X-axis represents the treatment groups. (* is a significant difference observed when compared to control, progesterone and leptin respectively from left to right)

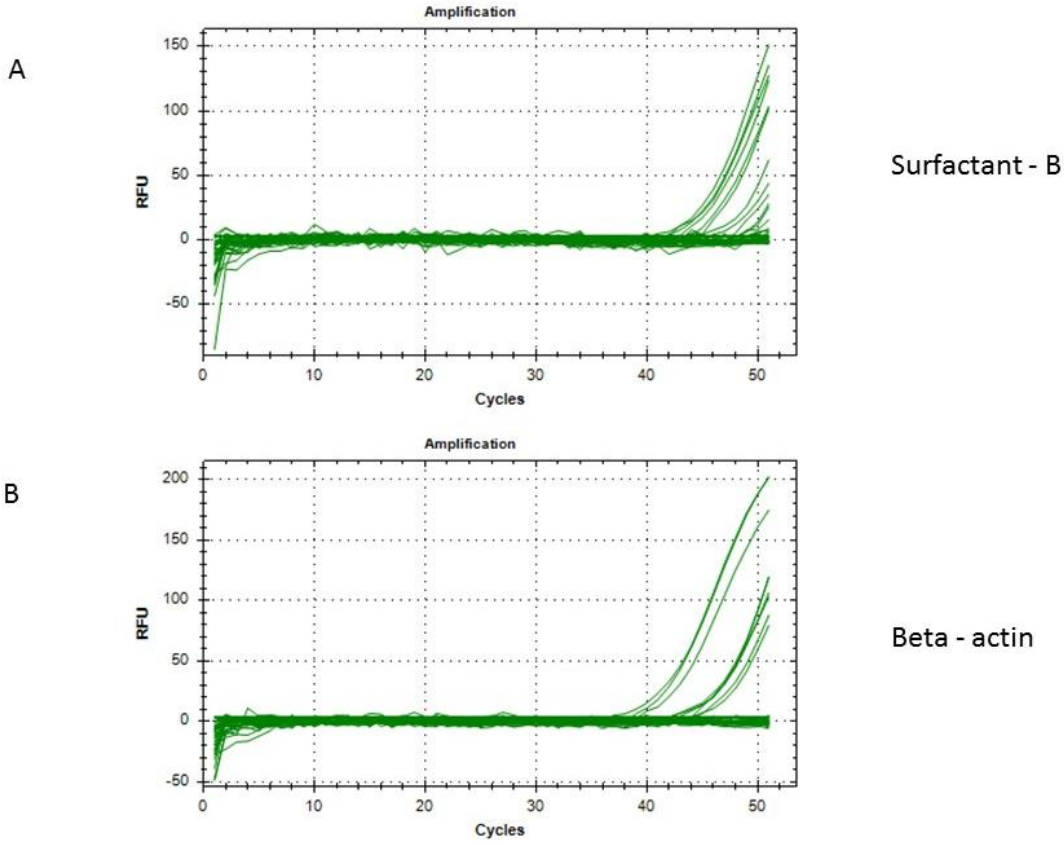


Figure 21: Image obtained through Real time PCR analysis for quantification of expression levels and mRNA secretion of surfactant-B by addition of 48 hours of control, betamethasone, progesterone, progesterone in combination with betamethasone (Samples 1.1 -4.3 as shown in the plate setup, analyzed in triplicates) A. Shows curves obtained with Surfactant-B primers and B. Shows curves obtained with Beta-Actin primers.

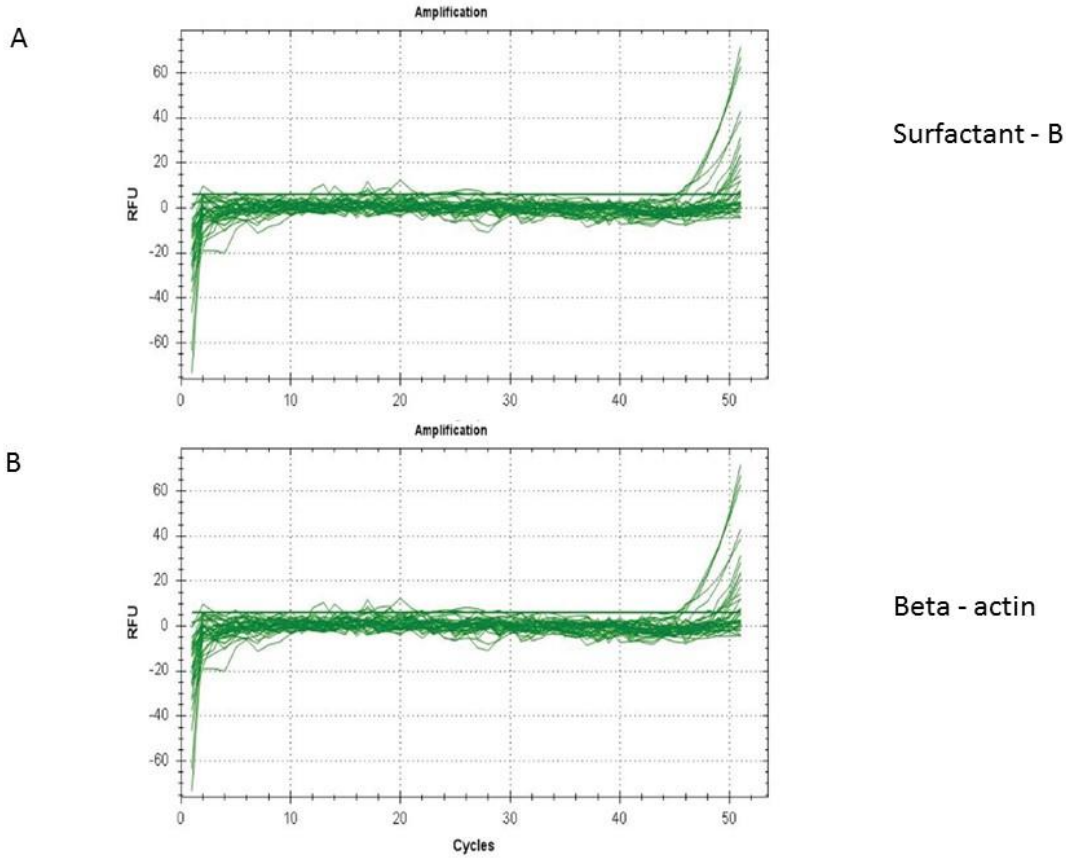


Figure 22: Image obtained through Real time PCR analysis for quantification of expression levels and mRNA secretion of surfactant-B by addition of 48 hours terbutaline, terbutaline in combination with betamethasone, leptin alone and in combination with betamethasone (Samples 5.2 -8.3 as shown in the plate setup, analyzed in triplicates) A. Shows curves obtained with Surfactant-B primers and B. Shows curves obtained with Beta-Actin primers.

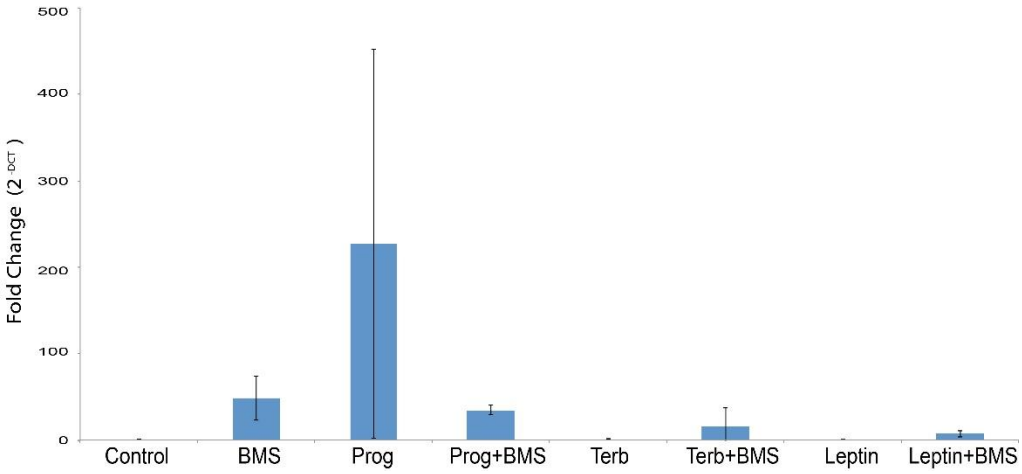


Figure 23: This image above represents the 48 hour of drug addition and the DCT analysis of qRT-PCR results for lung cells treated with different tocolytics. Here the Y-axis represents the relative change according to the $2^{\wedge DCT}$ calculations and X-axis represents the treatment groups. The actin did not have a valid CT value in most cases to accurately allow a DDCT analysis. The results represent the mean ± SEM from three independent wells of cells.

ΔΔCT analysis was performed for only the 24 hour calculation of expression levels and mRNA secretion in the human alveolar type-II cells, as there was no ß - actin available for the comparision in case of the 48 hour tests. The house keeper gene has not shown any variability in the peaks nor have shown good peak unlike the primers for Surfactant B have shown, for comparative quantification the surfactant B peaks with the ß-actin. So, for this reason we have calculated the ΔCT calculation which compares the surfactant – B peaks with the other drugs.

Discussion

Oftentimes, one of the first steps to testing the safety and efficacy of a drug or a combination of drugs is to screen them using an *in-vitro* cell culture system, or cell line. While it is known that cell lines are not an exact match to 'normal' or primary cells, they do often share adequate similar characteristics with primary cells that allows a first pass mechanism for screening. In the project presented here, we have used a human lung adenocarcinoma epithelial cell line, NCI-H441, as a reliable *in vitro* model to test three drugs that are commonly used to either help in fetal lung maturity, delay the contractions of labor and preterm birth or both (33, 34, 35). These drugs are betamethasone, a corticosteroid that is used to mature fetal lungs; progesterone, a steroid hormone that is used to slow contractions, and terbutaline, a beta-agonist that is purported to slow contractions and also mature the lungs. We also have tested a peptide hormone that is secreted by adipose tissue, i.e., leptin, for its ability to mature lungs and increase surfactant production. The goal of using the above-mentioned drugs in preterm birth is to slow the contractions for one to two days in order to allow the betamethasone to work in increasing surfactant secretion from the lungs of the fetus. The overall idea is that after administration of these drugs, birth of the infant can occur and respiratory distress syndrome can mostly be avoided. However, few studies have been performed to determine the effect of some of these drugs on the lung cell production of surfactant and lipids. Therefore, the main aim of this project was to determine if each of the drugs would

cause an increase in surfactant production in the cells and lipid secretion from the cells. We tested each drug alone and in combination with betamethasone in our cell culture model using: High Performance TLC analysis of lipids secreted; immunocytochemistry and western blotting for surfactant-B production; and Real Time PCR to analyze the surfactant-B mRNA levels after treatment.

Betamethasone stimulates lipid secretion and surfactant-B production

We first tested whether betamethasone would stimulate lipid secretion and surfactant-B production in this NCI-H441 cells. HPTLC analysis revealed that betamethasone stimulated lipid release from the cells at 24 hours. The results also suggest that the top band was most probably lecithin, since it ran on the TLC plate at a similar retention value (R_f) compared with the standard for lecithin run at the same time. Furthermore, it has previously been reported that under similar running conditions, lecithin ran ahead of sphingomyelin, which does correlate with our results of the standards (68). However, the identity of the bands will require further analysis by mass spectrometry to determine the true identity of each band, as either authentic lecithin or sphingomyelin, or their metabolites. Finally, secretion of the top band was consistently stimulated by betamethasone whereas, the other bands were less stimulated. The immunocytochemistry strongly supported the betamethasone effect by showing a very large increase in surfactant-B immunostaining after administering it to the cells. In addition, Real Time PCR showed a similar trend where surfactant B mRNA was stimulated with the addition of betamethasone. These results are similar to other studies that showed this cell line reacted similarly by causing an increase in expression levels of SP-B mRNA (69).

In contrast, the western blot analysis of cellular surfactant-B did not show a significant increase after addition of betamethasone. The large variability in the western blot data could possibly be explained in several ways. First the only protein band we observed was at ~38 kDa, which corresponds to the full-length size of pro-surfactant-B. However, the antibody was not designed to detect the 9 kDa band of mature surfactant B. If surfactant-B is stimulated to overproduce and only a small amount of the pro-form of surfactant-B is cleaved to the mature form, it may be difficult to detect a change even in the larger pro-form. Likewise, secretion of mature surfactant-B and pro-surfactant-B may have caused a significant decrease in the signal in the cell lysates. Preliminary experiments results using the media from the cells at the time of harvest did not show a 38 kDa band for surfactant-B protein nor a band for the mature form, suggesting that both forms might not be secreted. A more thorough analysis of the cell lysate and media protein content using ELISA for the mature 9kDa peptide would provide a better picture of the effects that betamethasone has on these cells.

In contrast, at 48 hours, there was a general higher secretion of lipids including from the control cells to the point that there was no significant Betamethasone stimulation. The immunocytochemistry and western blots results confirmed these results, though again, there was too much variability to make a definite conclusion. Real Time PCR was not conclusive as many of the PCRs failed due to low RNA extraction from the cells at 48 hours.

Progesterone

Progesterone is commonly used to slow contractions due to its effect on suppressing the calcium-calmodulin-myosin light chain kinase system by reducing the

calcium influx into the epithelial membrane and altering the resting potential of smooth muscle (45). In our study, we found that progesterone had a significant effect on lipid secretion, compared to control cells. In particular, the top, presumably lecithin band in the HPTLC plates appeared to be stimulated with progesterone alone, and was augmented with betamethasone. Likewise, we observed that the immunocytochemical staining was very similar between progesterone and progesterone in combination with betamethasone. The Real Time PCR analysis of the surfactant B mRNA expression was low in progesterone treated cells when compared to controls but was significantly increased with the addition of betamethasone in combination with progesterone. These results are similar to previous studies that there was a marked increase in surfactant proteins due to progesterone (43). As with the control and betamethasone alone treated cells before, western blots failed to show significant differences for progesterone and progesterone/betamethasone treated cells. At 48 hours there appeared to be no significant difference in lipid secretion, surfactant B staining or proteins on western blots.

The results from our study suggest that progesterone in conjunction with betamethasone could be a useful and effective treatment to both slow labor contractions and stimulate lung maturation. Furthermore, the results suggest that progesterone in combination could be a better short term, i.e., 24 hours, treatment.

Terbutaline

Terbutaline is an interesting ‘dual function’ drug because it relaxes the smooth muscles to delay contraction and it is also used as a treatment for asthma as a beta-agonist. Terbutaline’s effect on lipid secretion, especially band 1 lecithin, was slightly higher or almost similar to that of progesterone’s at 24 hours. This result is supported by

a study showing an increase in phosphatidylcholine (Lecithin) secretion in alveolar type II cells (50). Immunocytochemistry showed an increased staining of SP-B after administration of terbutaline both with and without betamethasone. Real Time PCR was less satisfactory with a very large variability that made it impossible to draw any conclusions. However, it appeared that there was a significant decrease in surfactant-B mRNA expression between terbutaline + betamethasone and progesterone + betamethasone at 24 hours of treatment. As usual, western blot analysis failed to show a difference due to the high variability of the bands and β-actin staining. The results at 48 hours were similar to those at 24 hours with the exception that band 2 in the lipids was significantly increased with betamethasone. While we have considered band 1 of the HPTLC plates to be lecithin, we had not made a determination about the other bands, i.e., bands 2, 3, and 4, though we propose that band 3 is sphingomyelin. With terbutaline treatment band 2 appeared to be significantly increased, and could have been a metabolite of either lecithin or sphingomyelin. The difference in band 2 at 48 hours on HPTLC and bands 1, 2, and 4 at 24 hours are consistent with previous reports of terbutaline stimulating lecithin secretion and also increased expression of surfactant lipids as a whole potentially due to the beta-agonist function of terbutaline, and not as a stimulator of synthesis (50, 70). Terbutaline is considered a very short term treatment, i.e., <48 hours, and our results suggest that that it does have effectiveness at this time point.

Leptin

The first question about leptin was whether there are leptin receptors in the lung cells. There have been reports that different receptor isoforms are expressed in the lungs, and that leptin itself is also expressed. We tested for the presence of the leptin receptor by

western blot analysis in each of the samples from the stimulation experiments, as some recent studies have confirmed the expression of leptin receptor by fetal rat lung type – II cells and also by the NH441- human adult lung epithelial cells (73). Although there was a weak band in the western blots, we were able to determine that there was a leptin receptor in the lung cells. Leptin receptor protein was found at 150 k Da and 100 kDa predominantly in many experiments, also showing a weak band at 75 kDa for some of the drug treated cell lysates. These protein bands when probed by a specific anti-leptin receptor protein primary antibody suggests that the receptor might have been post-translationally modified by an enzymatic cleavage or have undergone degradation. Future experiments will determine if another receptor isoform predominates in these cells.

Although leptin is not considered a tocolytic, it has been proposed to have an effect on lipid and surfactant secretion in the lung. In our study, leptin caused a profound stimulation on the secretion of lipids, specifically bands 1 and 2, after 24 hours of treatment. This result was similar to the result obtained by Torday et al showing that leptin plays a role in stimulating the *de nevo* synthesis of surfactant phospholipid particularly in H441 cells and also fetal rat lung type II cells (73). Cellular staining of SP-B also appeared to be brighter with leptin as well as with leptin + betamethasone. The RealTime PCR results showed a very dramatic decrease in mRNA at 24 hours with leptin treatment when compared to betamethasone, but not a significant decrease in mRNA with when compared to control. However, the combination of leptin and betamethasone did appear to cause stimulation of surfactant B mRNA, though it was still low. These results suggest that leptin may play a role in surfactant gene regulation in the lungs. In previous studies, it has been shown that the expression of surfactant – B protein in some cell lines

including NCI441 cells was modestly increased by the presence of glucocorticoids and also that the expression of SP-B gene would primarily be controlled at the post-transcriptional level than directly by the gene transcription (74, 75, 70, 71). It also suggests that women who are producing a large amount of leptin naturally may also be suppressing the surfactant gene expression in the fetus and hence there is a propensity for problems associated with obesity as well as having a lower body weight or BMI. Thus, leptin may be playing a larger role than first previously thought. This is similar with results obtained using rat primary alveolar cells that also showed a reduction in surfactant B mRNA and protein by western blots (72, 73). In another study, leptin was found to cause an increase in fetal lung cell surfactant B mRNA and protein (18), though it appeared to be temporally regulated to a very small window of gestation (52, 76). The authors postulated that other cell interactions, e.g., clara cells, may be involved in regulating the expression of surfactant B in response to leptin (18). It has also been proposed by Waddell et al (2003) that the plasma leptin binding sites may occasionally contribute to restrict the access of leptin to target tissues, suggesting that the soluble leptin receptor isoform may sometimes effect the transportation and metabolism of leptin, during pregnancy (77 – 79). Another study has postulated that androgens might contribute to inhibit the surfactant production with an addition of leptin, which backs up a slight decrease of SP-B mRNA in the study when compared to betamethasone treated cells, as we have used adenocarcinoma type – II cells of male adult obtained from ATCC (80).

Western Blot Problems

There was a small difference in the protein content of β-actin when compared to the specific-protein (surfactant-B). This is due to the fact that the same PVDF membrane has been used for detecting the protein bands of surfactant-B and then stripped with a stripping buffer for analyzing for the total protein content. Though we are aware that stripping procedure causes a slight decrease in the protein content, by stripping some of the protein content along with the antibodies. They have been stripped to detect the proteins at different molecular weights upon the same PVDF, so that we can exactly prove that the proteins are specifically detected with the addition of their specific antibodies (to confirm that they are not non-specific proteins). It is interesting to note that surfactant B is a 40 kDa pro-protein that is cleaved intracellularly to a 9 kDa peptide that can homodimerize with itself. This combination would give a peptide of 18kDa. We did not observe any of the 18 kDa peptides on the western blots, suggesting that the surfactant B was mostly in its pro-form. We have also never observed a smaller surfactant B peptide, i.e., 9 kDa in these cells, further suggesting that the enzyme that cleaves it is either missing defective or very inactive.

Conclusions

The objective of this study was to determine if adult human lung cells could be used to study the effects of different drugs on lipid secretion and surfactant-B production. We have shown results similar to previous reports that lipid secretion does respond to betamethasone, progesterone, terbutaline, and leptin in these cells. Furthermore, mRNA levels and immunocytochemistry appeared to confirm the lipid profile suggesting that these therapies may be used as previously reported. Our results support the continued use of progesterone alone as a tocolytic or in conjunction with betamethasone for its ability to help mature the lungs. However, the results also suggest that leptin may play a role in regulating surfactant production and surfactant mRNA expression that has previously been unexplored. Further research in this area as well as the longer-term use of terbutaline is necessary to provide insight into the mechanisms for these types of drugs and as a precursor to designing and testing better tocolytics in the future.

Future studies

It appears that all of the tocolytics except progesterone in combination with betamethasone, may be further tested before performing future clinical work. Factors potentially affecting the mechanisms involved in the functioning of these drugs should be identified including genetic factors, growth factors and inhibitory mechanisms which are coming into play during the development of the lung. In addition, *in vivo* effects of these drugs on surfactant production should be identified for a thorough mechanistic explanation.

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