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The Effects of Progesterone Induced Blocking Factor and 17-Hydroxyprogesterone Caproate on the Pathophysiology of Preeclampsia

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THE EFFECTS OF PROGESTERONE INDUCED BLOCKING FACTOR AND 17-HYDROXYPROGESTERONE CAPROATE ON THE PATHOPHYSIOLOGY OF PREECLAMPSIA

by
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ABSTRACT

Preeclampsia (PE) is responsible for about 20% of the 13 million preterm births each year worldwide, including 100,000 cases annually in the United States. Despite being a leading cause of maternal and perinatal morbidity, the mechanisms of pathogenesis are still largely unknown. PE is progesterone deficient state characterized by hypertension, chronic immune activation, endothelial dysfunction and severe forms can lead to seizures. Treatment of seizures includes the administration of magnesium sulfate ($MgSO_4$) though not all PE patients are responsive, and it does not decrease PE-associated hypertension. To resolve these conditions, PE patients are delivered early thereby making PE the leading cause for fetal mortality and morbidity worldwide. Hypothesizing that the reduced progesterone levels accounted for the onset of hypertension in PE, the effects of both 17-hydroxyprogesterone caproate (17-OHPC) and Progesterone Induced Blocking Factor (PIBF) were studied in hypertensive pregnant rat models of PE. Following the intervention, blood pressure and endothelin-1 were reduced while nitric oxide was elevated.

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LIST OF ABBREVIATIONS

PE	preeclampsia
sFlt-1	soluble fms-like tyrosine kinase-1
17-OHPC	17-hydroxyprogesterone caproate
PIBF	progesterone induced blocking factor
ET-1	endothelin-1
NO	nitric oxide
TH2	T-helper type 2
$MgSO_4$	magnesium sulfate
PPET-1	preproendothelin-1
VEGFR1	vascular endothelial growth factor receptor 1
VEGF	vascular endothelial growth factor
IL-4	interleukin 4
NK	natural killer
NP	normal pregnant
RT-PCR	real-time polymerase chain reaction
HUVECs	human umbilical venous endothelial cells

INTRODUCTION

Preeclampsia (PE) is a multifactorial hypertensive disease that affects and complicates up to 8% of pregnancies in the United States and 12% in Mississippi and is responsible for one-fifth of all the preterm births in the United States annually[5,7].

While PE results in high levels of maternal and perinatal morbidity, the only treatment currently available is preterm delivery[16]. To allow for the development of mechanism-based therapies to better manage patients with PE, efforts to identify the key components in the pathogenesis of PE at the molecular level are currently underway. Findings from these prior studies have discovered that PE is associated by decreased levels of progesterone, new onset hypertension, chronic inflammation, mitochondrial dysfunction, and endothelial dysfunction[2] (Figure 1). These PE-associated changes are typically diagnosed near the 20th week of pregnancy to third trimester during pregnancy.

During the third trimester of a normal pregnancy, progesterone levels are measured to be about 34 ng/mL[9]. In pregnant women with PE, this increase is not observed (15 ng/mL; Figure 2). A key signaling molecule during pregnancy, progesterone has the potential to impact a number of physiological processes in pregnant women. As part of regulating the progesterone-dependent pathways, the body releases factors like Progesterone Induced Blocking Factor (PIBF) which helps to control proinflammatory mechanisms that occur early in gestation. PIBF then goes on to stimulate Interleukin 4 (IL-4) and therefore increase Th2 cytokines. IL-4 and Th2 cytokines are thought to promote a more tolerant, anti-inflammatory environment that protects the fetus. PIBF in women without PE, also helps to crease cytolytic natural killer (NK) cells as well as Th1 cytokines (Figure 3, 4). Thus if, progesterone is decreased then likewise, so is PIBF

and these inflammatory processes are not appropriately regulated. 17-ortho hydroxyprogesterone (17-OHP) is a progesterone that is mainly produced by the adrenal glands, gonads, and ovaries (Figure 5). Clinically, 17-orthohydroxyprogesterone caproate (17-OHPC) is a synthetic progesterone that is currently utilized for treating preterm labor in pregnant women without PE. However, due to the lack of clinical data, it is not yet used as a treatment for PE[6, 13-14].

Mitochondrial oxidative stress refers to the production of more than normal amounts of reactive oxygen species (ROS) from the mitochondria. Circulating factors such as inflammatory cytokines and cells induce mitochondrial dysfunction in a preeclamptic placenta which can lead to a high instance of mitochondrial DNA damage as well as impaired function of the mitochondria in the electron transport chain[2].

Endothelial dysfunction is a condition in which the endothelial layer (the inner lining) of the small arteries fails to perform all of its important functions normally. It is characterized by increased levels of sFlt-1 and ET-1, as well as a decrease in levels of nitric oxide (NO). sFlt-1 is a soluble vascular endothelial growth receptor (VEGFR1). VEGF is important in maintaining the vascular endothelium but is antagonized by sFlt-1 during PE (Figure 6). Endothelin-1 is a potent vasoconstrictor while nitric oxide is a vasodilator[11]. This results in a strong correlation leading to increased levels of ET-1 and decreased levels of NO. This imbalance leads to chronic vasoconstriction.

During early stages of pregnancy complicated by preterm labor, but not preeclampsia, 17-OHPC is given to patients as early as 20 weeks[13,14]. 17-OHPC is delivered to these patients weekly in order to prolong labor and has increased time to delivery, yet there is no administration of 17-OHPC to patients afflicted with severe preterm preeclampsia.

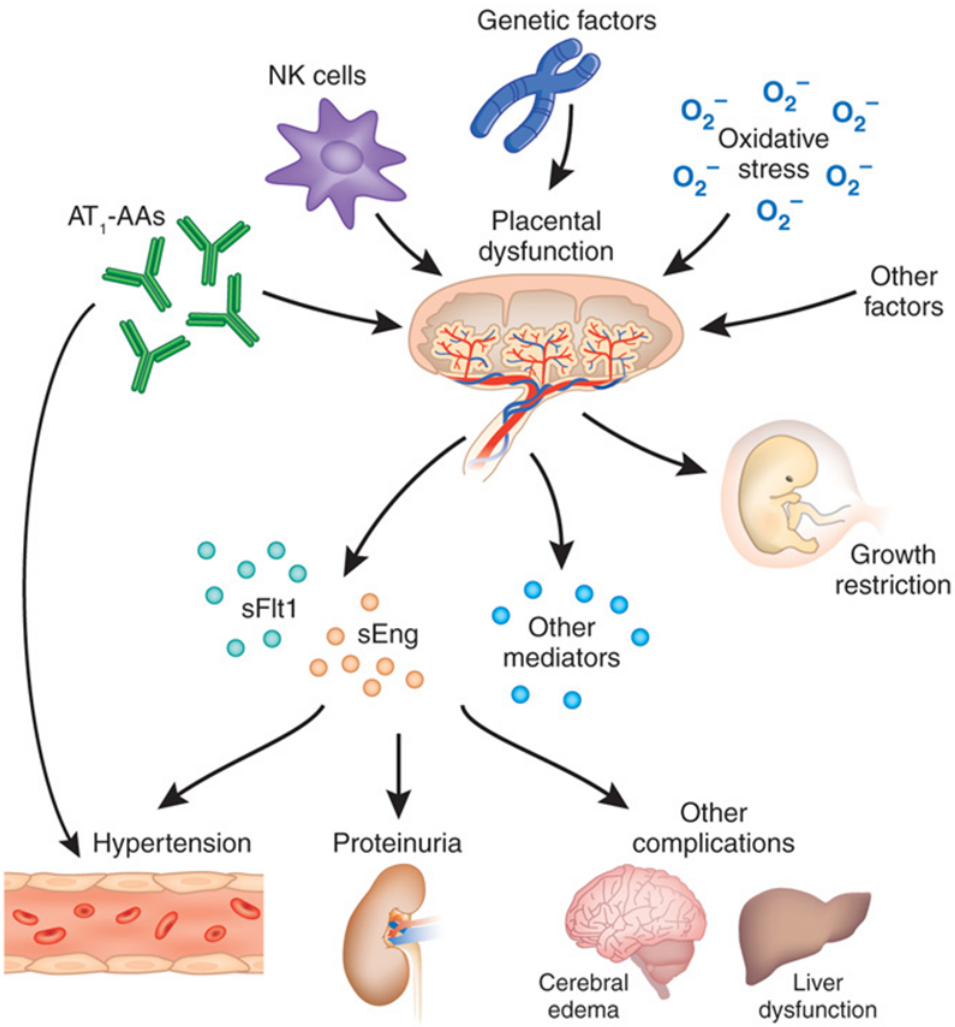


Figure 1: Factors Contributing to Preeclampsia[18]

There are several factors that contribute to placental dysfunction such as a genetic component, Angiotensin II Type-1 Receptor Autoantibodies, Natural Killer cells, oxidative stress, and various other factors. Placental dysfunction leads to an excess of sFlt-1 and soluble endoglin (sEng) as well as increased growth restriction of the fetal unit. The outcomes of this are hypertension, proteinuria, and various other complications. This comprehensive figure illustrates the multifactorial components leading to preeclampsia.

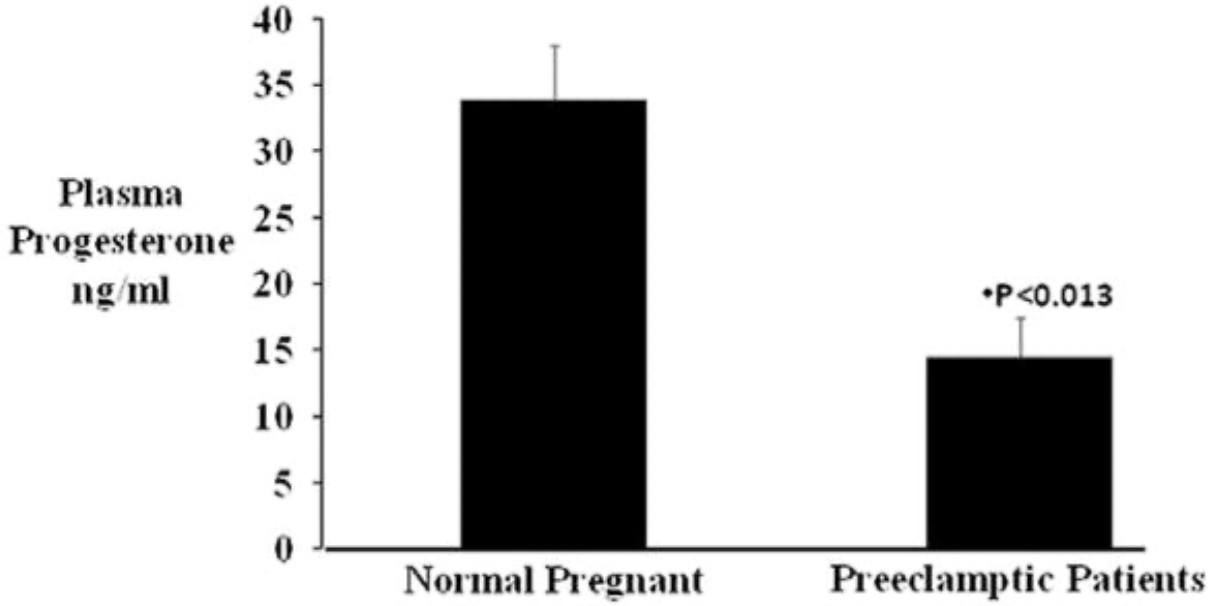


Figure 2: Progesterone Levels in Normal Pregnancy Versus Preeclampsia[9]

The graph above demonstrates the levels of circulating progesterone in a normal pregnancy versus during preeclampsia. In normal pregnancy, plasma progesterone levels are around 34 ng/ml, whereas preeclamptic patients have significantly lower levels ($p<0.013$) of about 14 ng/ml.

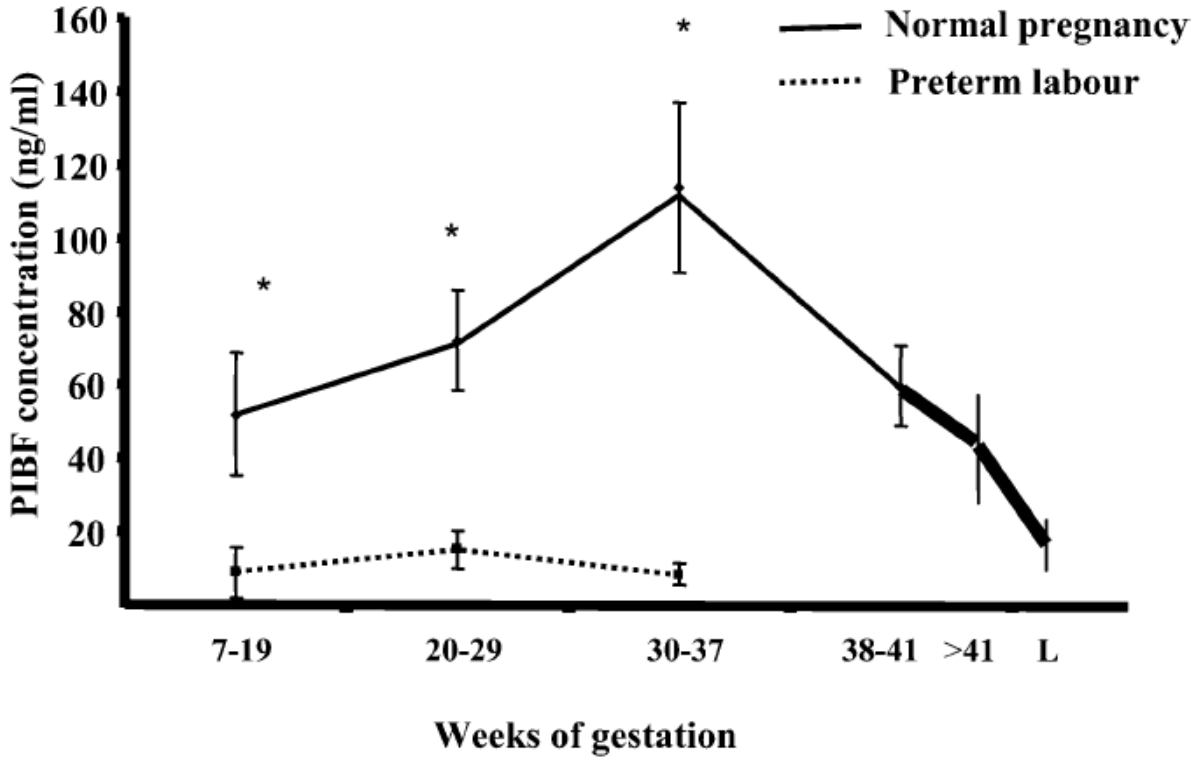


Figure 3: PIBF Levels in Normal Pregnancy Versus Preeclampsia[19]

Progesterone Induced Blocking Factor (PIBF) in normal pregnancy increases to level around 110 ng/ml in the 30-37-week period of gestation. During preterm labor, PIBF concentration is significantly lower with its highest concentration being around 10 ng/ml in the 20-29-week period.

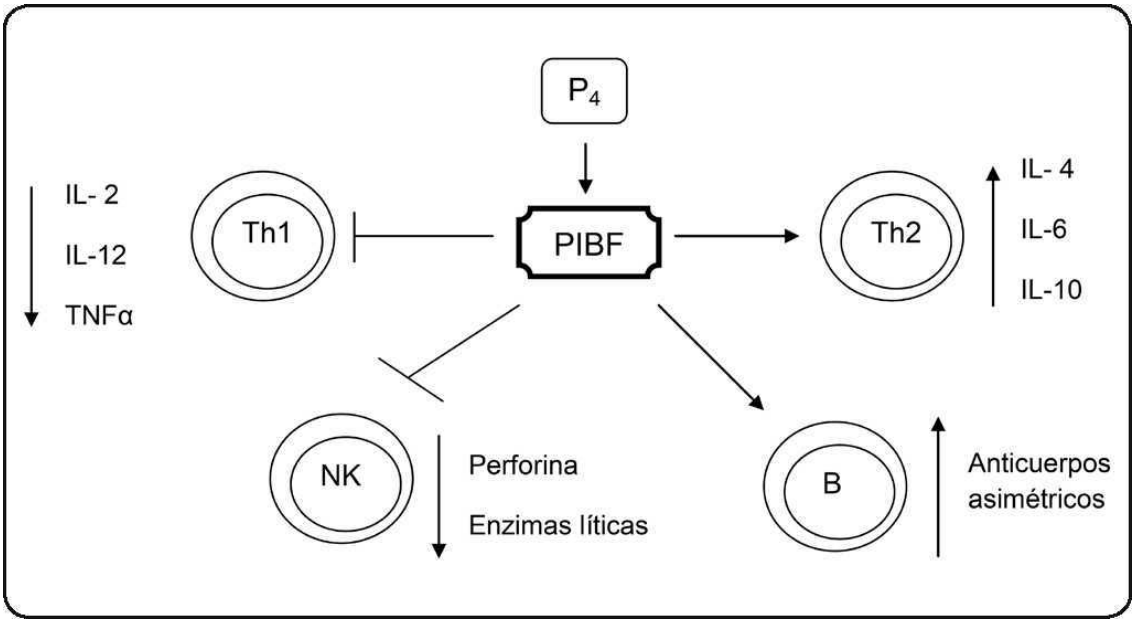
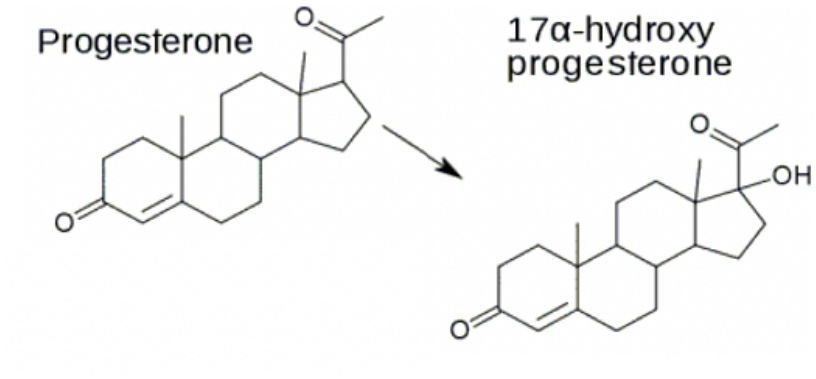


Figure 4: PIBF Diagram[10]

During pregnancy, progesterone activates Progesterone Induced Blocking Factor (PIBF), which goes on to initiate a series of events. PIBF inhibits T-helper type 1 (Th1) cells, lowering levels of interleukin-2 (IL-2), interleukin-12 (IL-12), and Tumor Necrosis Factor alpha (TNFα). PIBF also inhibits Natural Killer (NK) cells. T-helper type 2 (Th2) cells are activated which promotes interleukin-4 (IL-4), interleukin-6 (IL-6), and interleukin-10 (IL-10). PIBF also activates B cells, or B lymphocytes, to help with humoral immunity and the production of antibodies.



17-hydroxyprogesterone caproate

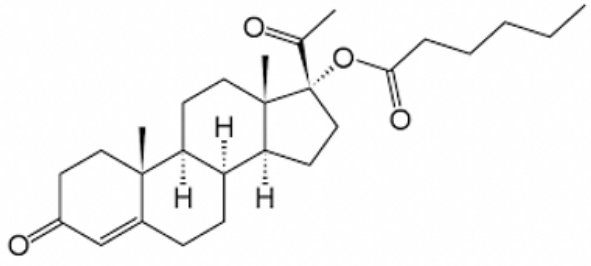


Figure 5: Progesterone and 17-OHPC Structures

The structural formulas shown are natural progesterone produced in the body, 17-alpha-hydroxyprogesterone with is a natural progestin, and 17-hydroxyprogesterone caproate which is a synthetic hormone.

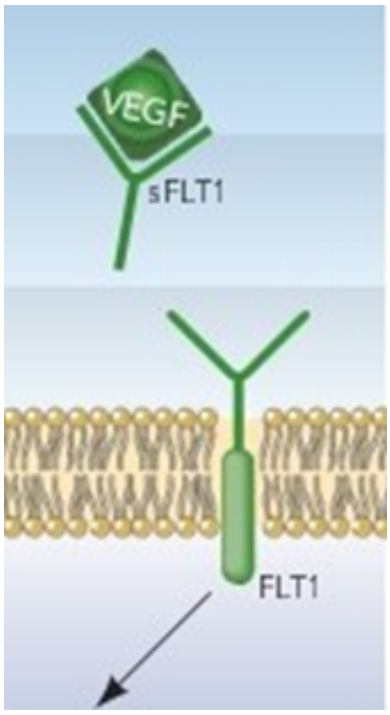


Figure 6: sFlt-1 and VEGF[22]

sFlt-1 is one of the main contributors to placental dysfunction. In a normal pregnancy, Vascular Endothelial Growth Factor binds to its FLT1 receptor. This, in turn, promotes angiogenesis, or the development of new blood vessels. During preeclampsia, an excess of the soluble fms-like tyrosine kinase receptor binds VEGF, creating an antiangiogenetic effect. This antiangiogenetic effect impairs relaxation of the blood vessels, leading to endothelial dysfunction.

METHODS

Inducing Hypertension via the sFlt-1 Model

The pregnant Sprague-Dawley rats used for this study were obtained from Harlan Sprague Dawley in Indianapolis, Indiana (Figure 7A). The rats were kept in housing conditions that consisted of a 23°C temperature room with access to food and water. Animals weighing 200-250g were randomly assigned to Normal Pregnant (NP; control) or NP + sFlt-1 groups. On day 13 of gestation, the NP + sFlt-1 group rats were put under anesthesia in order to place the sFlt-1 mini osmotic pump into the abdominal cavity to induce hypertension (Figure 7B). This pump dosage was designated as 3.7 $\mu m \cdot kg^{-1} \cdot day^{-1}$ for 6 days, the 6 days representing days 13-19 of the animals' gestation period. After the animals' abdominal cavities were sutured, analgesics were applied via subcutaneous injection of 5 mg/kg Carprofen once a day for 2 to 3 days. All experimental procedures performed in this study were executed in accordance with the National Institute of Health guidelines developed for the use and care of animals. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. Surgical procedures were carried out under the appropriate anesthesia and analgesics were give post-operatively as needed, under the supervision of the veterinary staff.

17-OHPC Administration

On day 15 and 18 of gestation, a group of NP + sFlt-1 treated rats were intraperitoneally injected with 17-OHPC. The solution dosage administered was 0.5 cm^3 solution of 3.32 mg/kg^9. The NP rat group were not treated with an injection of 17-OHPC because the previous studies demonstrate no effect on blood pressure or other molecular factors in NP rats[3] and is therefore seen as an unnecessary procedure for the rat.

PIBF Administration

On day 15 of gestation, a group of NP + sFlt-1 treated animals were intraperitoneally injected with PIBF at a dosage of 2.0 μm/mL. This dosage was chosen from previous experiments inducing hypertension with the Reduced Uterine Perfusion Pressure (RUPP) model. These experiments showed that the appropriate in vivo dosage was 2.0 μm/mL in order to decrease signs of preeclampsia in response to elevated sFlt-1 during pregnancy.

Mean Arterial Blood Pressure Measurement

On day 19 of gestation, the animals were placed under isoflurane anesthesia so carotid arterial catheters could be inserted in order to measure blood pressure. The catheters were placed into the carotid artery towards the back of the animal's neck to be made available externally. Blood pressure was then measured via a pressure transducer and recorded for 30 minutes after a 30-minute stabilization period. After measuring MAP, the animal's tissues were harvested, blood samples were collected, and pup and placenta weights were recorded.

Measuring Nitrate-Nitrite Levels

Plasma, obtained from the collected blood samples, was used in order to measure the circulating nitrate-nitrite levels using the Nitrate/Nitrite Colorimetric Assay Kit from Cayman Chemical (Ann Arbor, MI). Instructions that were induced were followed. The inter-assay coefficient of variation is 3.4% while intra-assay coefficient of variation is 2.7%.[2]

Measuring Renal Cortex Preproendothelin-1 mRNA Levels

In order to measure renal cortex preproendothelin-1 (PPET-1) levels, real-time polymerase chain reaction (RT-PCR) was used. The tissue samples from the kidney were stored in a -80°C freezer. Tissue samples were homogenized, and RNA was extracted using the RNeasy Protect Mini Kit from QIAGEN (Germantown, MD). A spectrophotometer was used in order

determine the concentration and the quality of the RNA. RT-PCR was performed using iQ SYBR Green Supermix and CFX96 Touch Real-Time PCR Detection System from Bio-Rad Laboratories (Hercules, CA). The primer sequences for PPET that were used are CTAGGTCTAAGCGATCCTTG forward and TCTTTGTCTGCTTGGC reverse.[2]

Measuring Mitochondrial Dysfunction

Placentas and renal samples taken from NP + sFlt-1 + PIBF animals were utilized in order to establish the peroxide production of the mitochondria. These sample were put into a mechanical homogenizer to lyse the cells. Next, the lysed cells are put through a centrifugation process in order to extract the mitochondria. After this, sample are put through an Oxygraph-2K to measure the amount of respiration or a fluorescence microplate reader in order to measure mitochondrial peroxide. An amplex red assay was used where samples were placed on a 96 well plate and put into a fluorescence microplate reader (Figure 8). The samples were incubated with a respiration buffer, superoxide dismutase, horseradish peroxidase, and succinate. Amplex red was added to start the reaction and the real-time production of peroxide was recorded. There were also wells without amplex red in order to serve as a control.

Measuring Mitochondrial ROS[23]

In order to determine the amount of mitochondrial reactive oxygen species, MitoSOX red dye was utilized. Serum obtained from the rats was used to determine mitochondrial ROS production with human umbilical venous endothelial cells (HUVECs). Human umbilical venous endothelial cells (HUVECs) were grown in HUVEC media and then cultured in a 6-well plate and incubated overnight, in order to determine the vascular endothelial cell mitochondria function. After incubation, these cells were washed and then incubated with MitoSOX red. Cells were then washed with Dulbecco's phosphate-buffered saline (DPBS) two times and incubated

again in a serum free medium for 4 hours. After this, flow cytometry was utilized in order to collect and analyze the cells. The basis of this assay is to determine if circulating factors mediate endothelial mitochondrial dysfunction.

Statistical Analysis

All of the data are expressed as mean ± SEM. Comparisons of control with experimental groups were analyzed by One-way ANOVA with Bonferroni multiple comparisons test as post hoc analysis. A value of $p < 0.05$ was considered statistically significant.[2]

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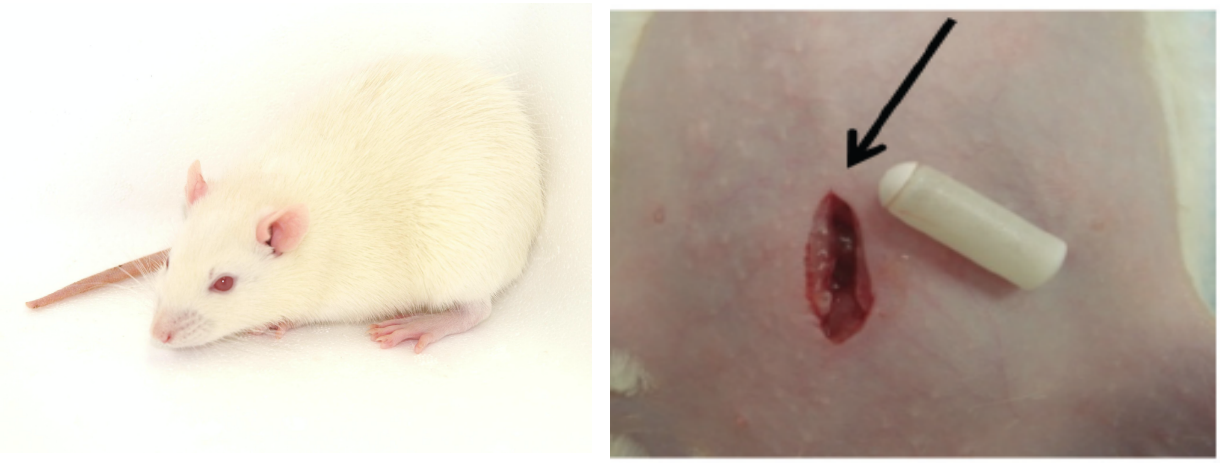

Figure 7: Sprague-Dawley Rat and Incision Site

For this experiment, pregnant Sprague-Dawley rats were obtained from Harlan Sprague Dawley in Indianapolis, Indiana (A). On the right, is a visual representation of the incision made in the rat's abdominal area in order to place the sFlt-1mini-osmotic pump (B).

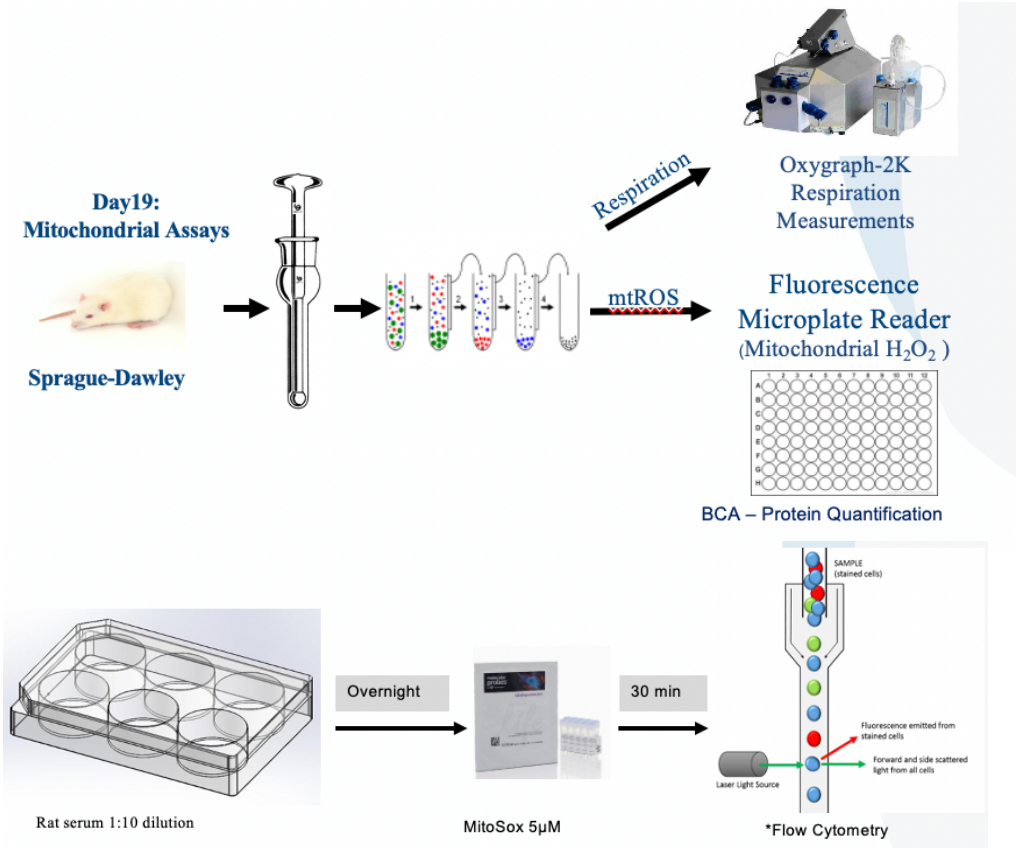

Figure 8: Mitochondrial Respiration and HUVECs Experiment Process

RESULTS

MAP was significantly increased in NP + sFlt-1 rats 117 ± 1 mmHg (n=13) compared to the NP control rats 99 ± 2 mmHg (n=12, $p < 0.05$). After administering 17-OHPC to these sFlt-1 treated rats, the MAP was significantly reduced to 102 ± 3 mmHg (n=8) (Figure 9). 17-OHPC showed no changes in either placental or pup weight in response to sFlt-1 or 17-OHPC during pregnancy (Figure 10). Recent study has shown that 17-OHPC, when given to NP rats, shows no change to either placental or pup weights[3].

In the NP + sFlt-1 treated rats, the renal cortex PPET-1, which is a marker for ET-1, showed a 3-fold increase compared to the NP animals. After administration of 17-OHPC, PPET-1 levels were significantly reduced (n = 5-7/group, $p < 0.05$) (Figure 11).

Plasma nitrate-nitrite levels were 44 ± 9 μM in NP rats (n = 9), 20 ± 3 μM in NP + sFlt-1 (n = 7) and were increased to 42 ± 11 μM in 17-OHPC treated rats[2] (n = 6) (Figure 11).

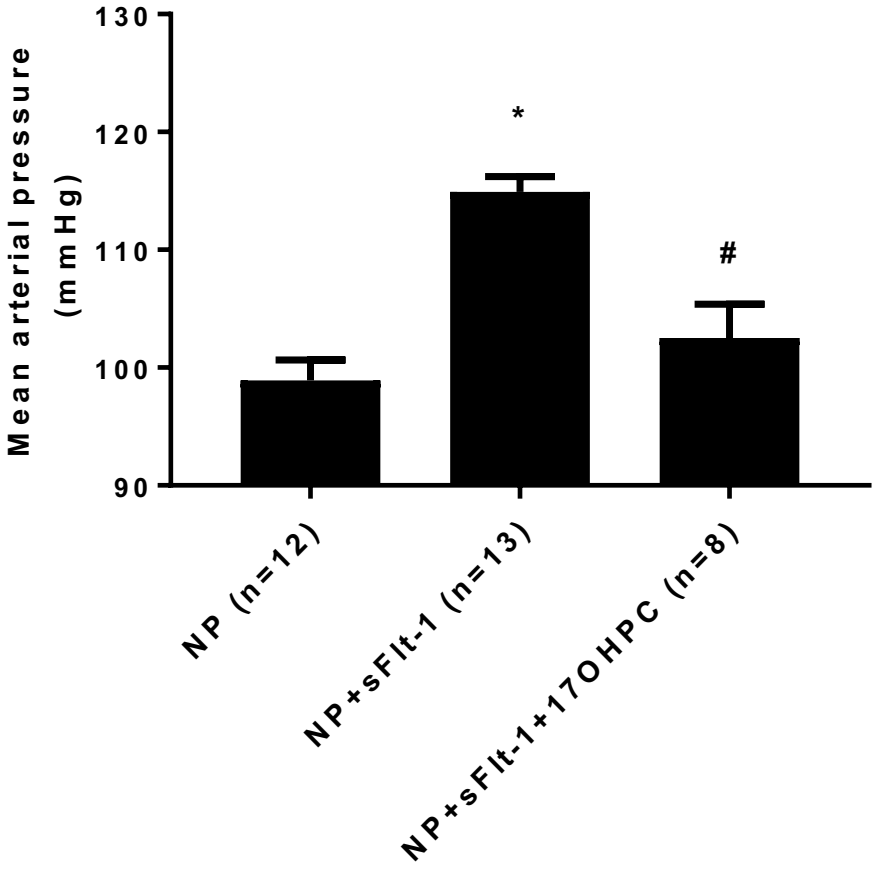

Figure 9: 17-OHPC Mean Arterial Pressure Graph[2]

Normal pregnant (NP) the mean arterial blood pressure (MAP) is 99 ± 2 mmHg (n=12). When sFlt-1 is administered, the blood pressure is significantly increased to 117 ± 1 mmHg (n=13) compared to the NP group ($p < 0.05$). When the sFlt-1 group was treated with 17-OHPC blood pressure was significantly reduced to 102 ± 3 mmHg (n=8) ($p < 0.05$).

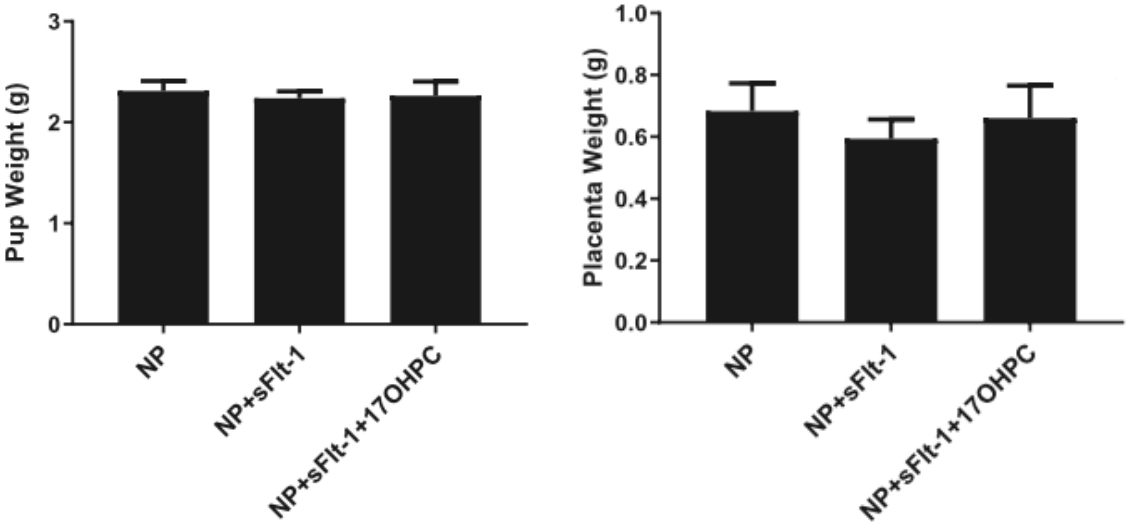

Figure 10: 17-OHPC Pup and Placenta Weight Graph[2]

Pup weight (left) did not differ between treatment groups: NP (n=12), NP + sFlt-1 (n=13), and NP + sFlt-1 + 17-OHPC (n=8). The graph of the right represents placenta weight in grams. No significant difference was observed in placenta weight either (right graph), although there is a slight upward trend between the sFlt-1 administered group (n=13) to the 17-OHPC treated group (n=8).

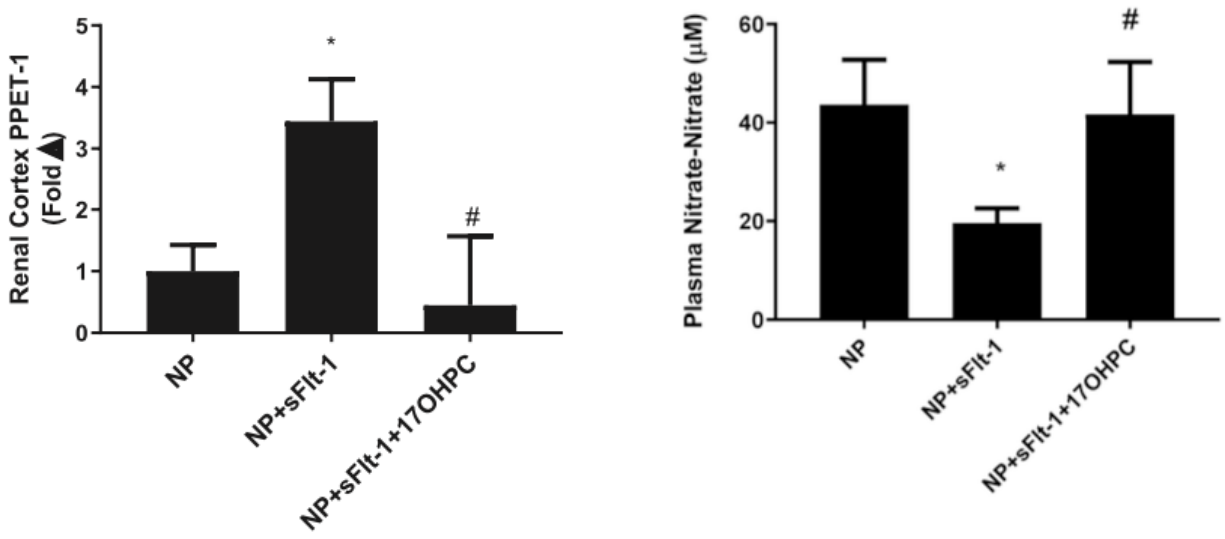

Figure 11: 17-OHPC Renal Cortex PPET-1 and Nitrate-Nitrite Graph[2]

Renal cortex preproendothelin-1 (PPET-1) levels were measured (left graph) and the sFlt-1 administered group showed a significant increase in PPET-1 compared to the NP group. When treated with 17-OHPC, PPET-1 levels were significantly lowered compared to the sFlt-1 group ($n = 5\text{-}7$/group, $p < 0.05$). Circulating plasma nitrate-nitrite was measured (right graph) with a level of 44 ± 9 μM in NP rats ($n = 9$), 20 ± 3 μM in NP + sFlt-1 ($n = 7$) and were increased to 42 ± 11 μM in 17-OHPC treated rats ($n = 6$).

Mean arterial pressure (MAP) was increased in s-Flt-1 rats to 112 ± 2 mmHg (n = 9) compared to control NP rats 98 ± 2 mmHg (n = 10, $p < 0.05$). Administration of PIBF reduced MAP to 100 ± 1 mmHg in the presence of s-Flt-1 (n = 9, $p < 0.05$) (Figure 12). There was also no change to pup weight between the NP, sFlt-1 treated, and PIBF treated animals (Figure 13). Body weights were NP rats (317 ± 9.32 grams), s-Flt-1 rats (322 ± 8.07 grams), and s-Flt-1 rats administered with PIBF (303 ± 6 grams).

PIBF was shown to reduce PPET-1 in response to elevated sFlt-1 during pregnancy, which was done by real-time PCR. In both the placenta and the renal cortex, there was an increase of endothelin-1 levels in the sFlt-1 treated rats compared to the NP rats. When treated with PIBF, these rats had a significant decrease in levels of endothelin-1 compared to the sFlt-1 treated rats (Figure 14).

The nitrate-nitrite levels in the sFlt-1 treated rats were significantly lower than the NP rats, but there was not a significant difference in the nitric oxide levels of the PIBF treated rats (Figure 15).

Placental mitochondrial state 3 respiration decreased in NP + s-Flt-1 infused rats (n = 3) (206 ± 67 pmol of O2/sec/mg, n = 4) compared to NP controls (n = 3) (268 ± 24 pmol of O2/sec/mg). State 3 placental respiration in NP + sFlt-1 rats administered PIBF (n=5) (309 ± 153 pmol of O2/sec/mg) was elevated compared to NP + sFlt-1 rats. Uncoupled respiration also decreased in NP + s-Flt-1 infused rats (n=3) (87 ± 42 pmol of O2/sec/mg, n=4) compared to NP controls (n=3) (164 ± 21 pmol of O2/sec/mg, n=3) and NP + sFlt-1 rats administered PIBF (n=5) (199 ± 93 pmol of O2/sec/mg, n=5) (Figure 16).

No effect was shown on renal mitochondrial reactive oxygen species and respiration with PIBF treated rats (Figure 17).

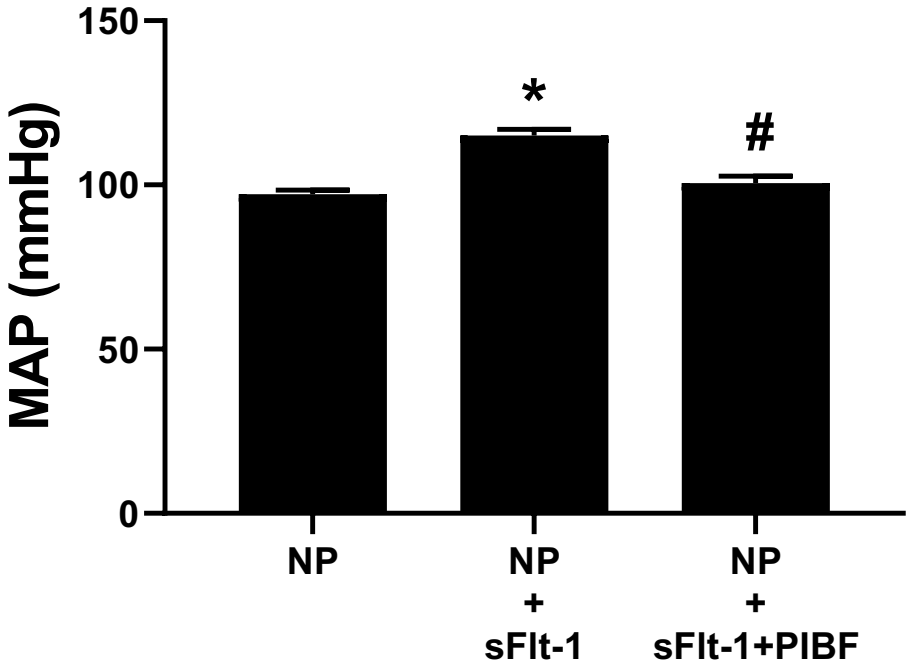

Figure 12: PIBF Mean Arterial Pressure Graph

Mean arterial pressure (MAP) for the PIBF treated rats. The sFlt-1 group showed significant increase in blood pressure at to 112 ± 2 mmHg (n = 9) compared to the NP group 98 ± 2 mmHg (n = 10, $p < 0.05$). The PIBF treated group indicated a significantly lower MAP compared to the sFlt-1 group at 100 ± 1 mmHg (n = 9, $p < 0.05$).

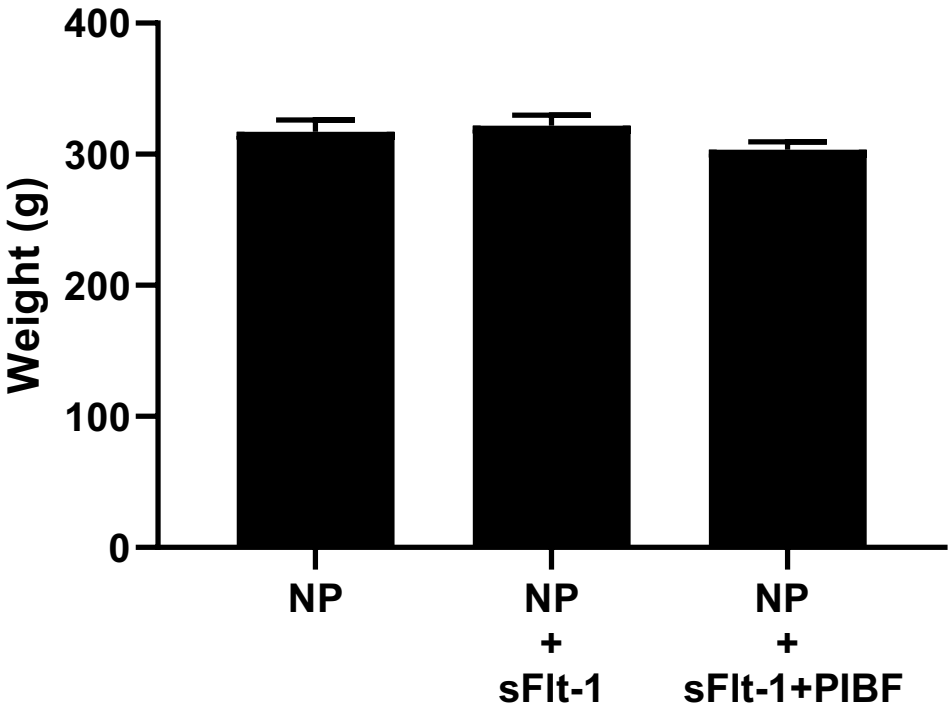

Figure 13: PIBF Pup Weight Graph

There was no difference in pup weight from the normal pregnant, sFlt-1 administered, and PIBF treated groups.

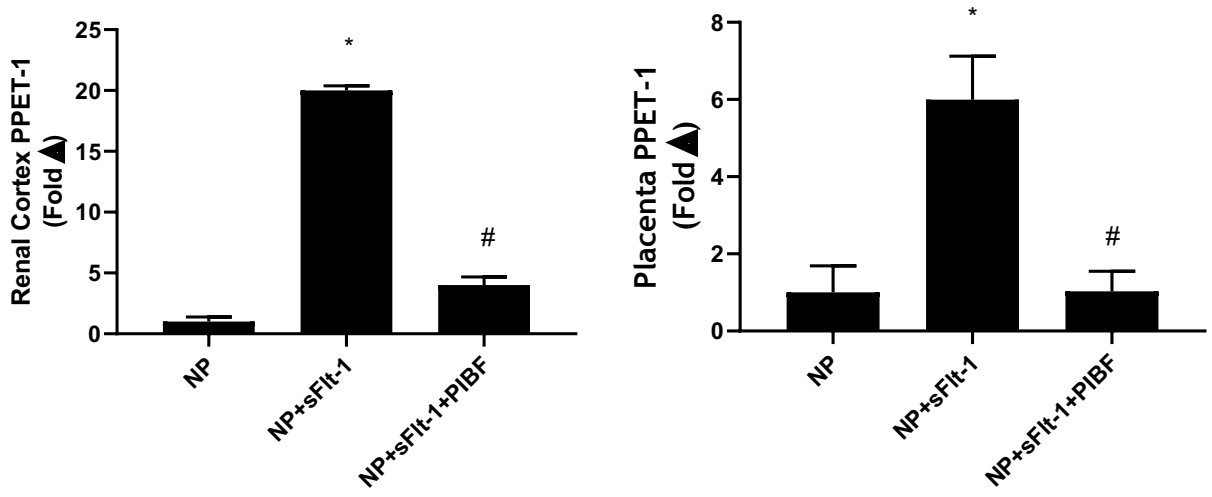

Figure 14: PIBF Placenta and Renal Cortex PPET-1 Graph

Placenta PPET-1 was measured in the graph to the right. In the sFlt-1 administered group significantly high levels of PPET-1 were seen as compared to the normal pregnant group. When treated with PIBF, PPET-1 levels were decreased compared to the sFlt-1 group. On the left graph, renal cortex PPET-1 levels were measured. When the rats were given sFlt-1 the PPET-1 levels were increased significantly from the NP group. When treated with PIBF, the levels of PPET-1 declined.

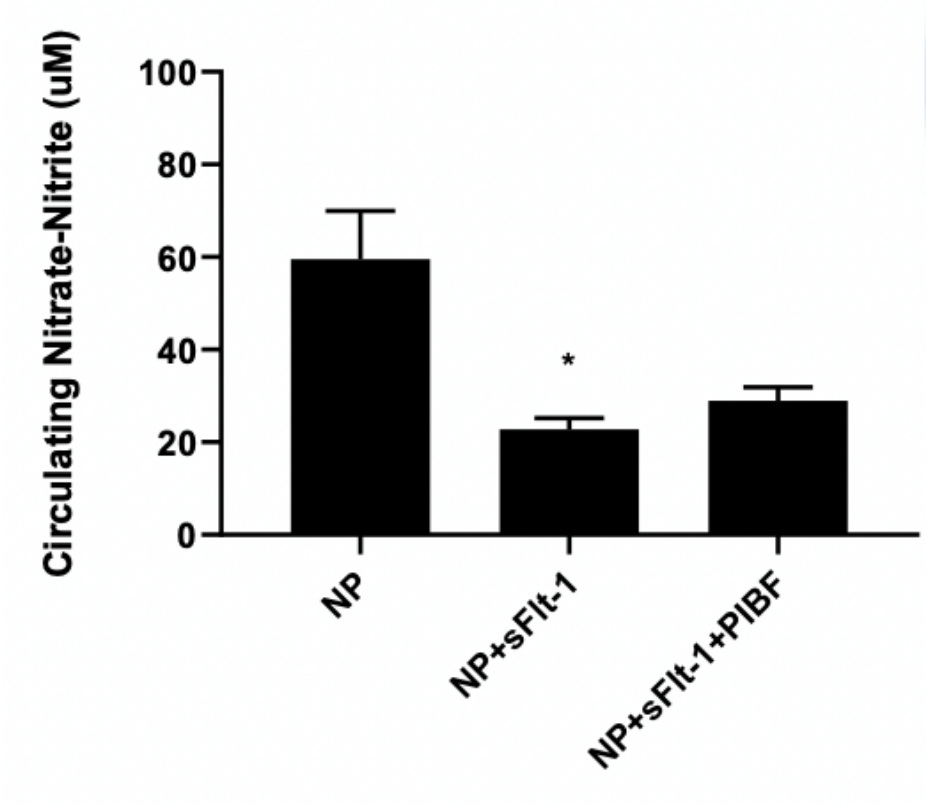

Figure 15: PIBF Circulating Nitrate-Nitrite Graph

A significant decrease was seen in circulating nitrate-nitrite in the sFlt-1 group compared to the normal pregnant group. When looking at the PIBF treated group, no change was seen compared to the sFlt-1 group.

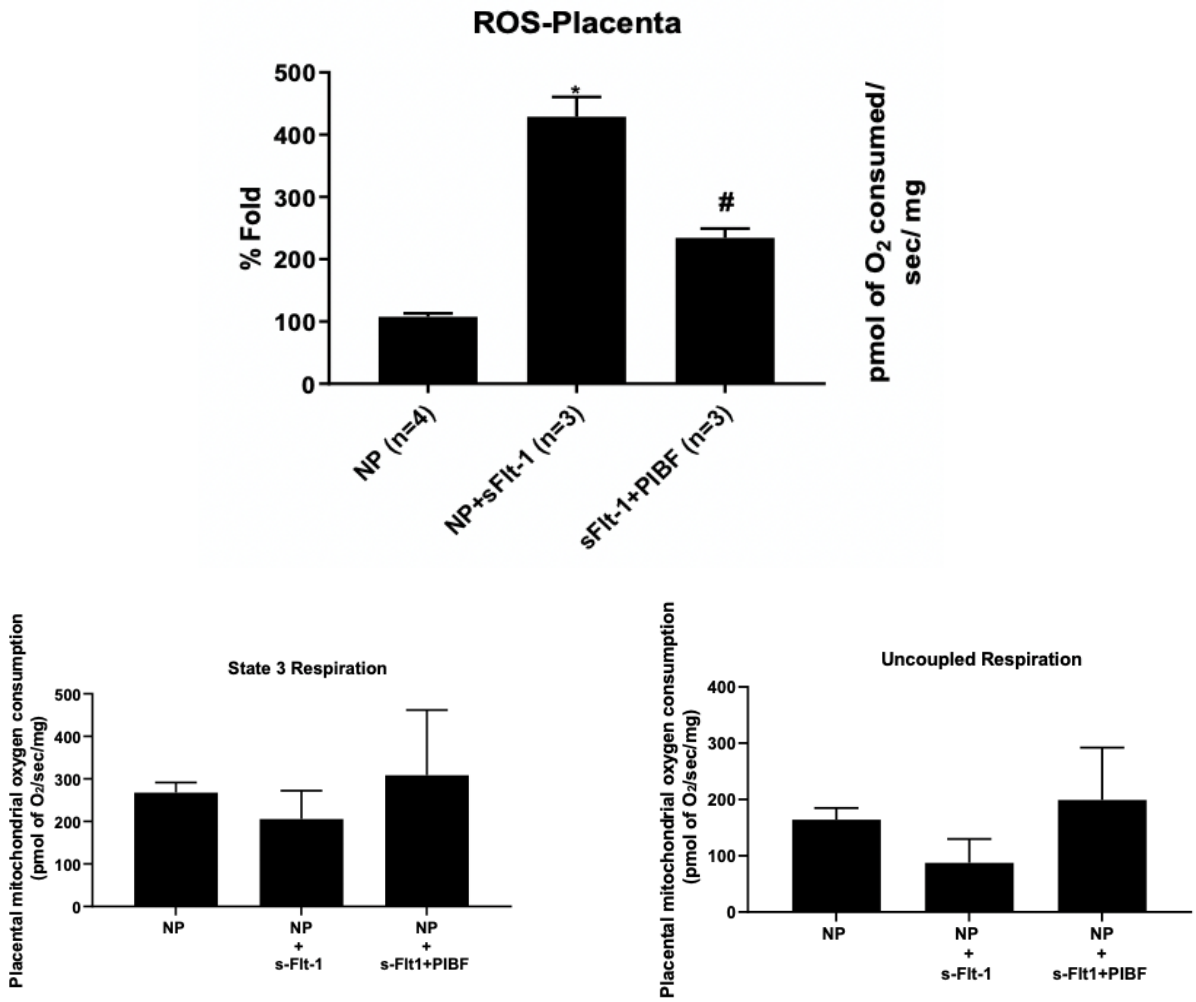

Figure 16: PIBF Placenta Mitochondria ROS and Respiration Graph

For placental mitochondria Reactive Oxygen Species (ROS; top graph), in the sFlt-1 group there was an increase in ROS compared to the NP group. The PIBF treated group displayed a significant decrease in ROS contrasted to the sFlt-1 group. No significant difference in placental mitochondria respiration (bottom right/left graphs) was seen, although the data suggests that respiration in State 3 could increase.

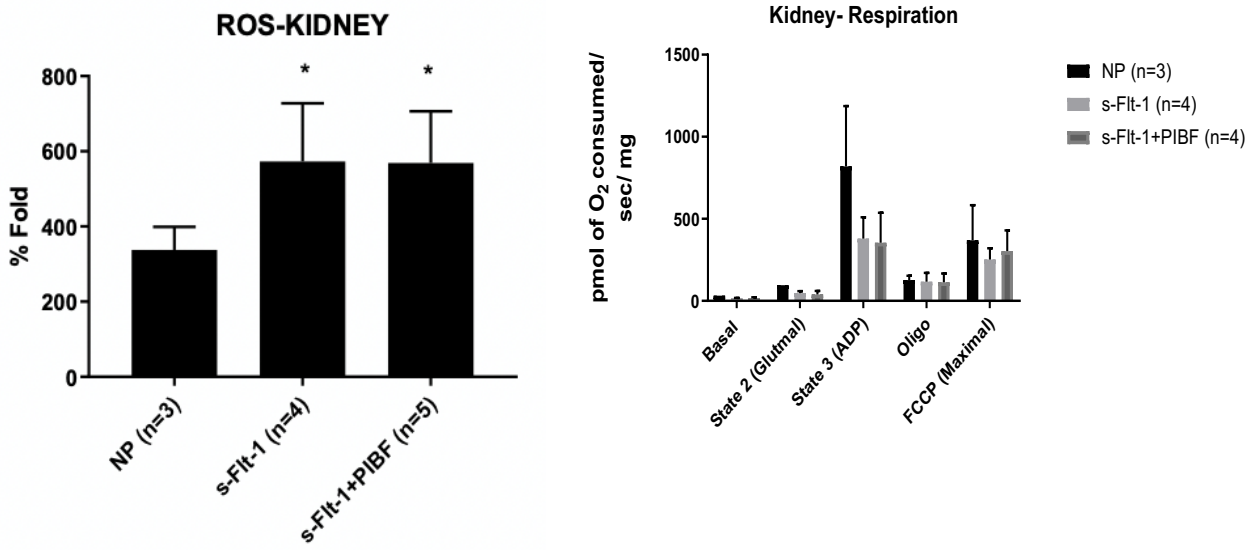

Figure 17: PIBF Kidney Mitochondria ROS and Respiration Graph

PIBF was shown to have no effect on renal mitochondria reactive oxygen species or renal mitochondria respiration.

DISCUSSION

A crucial part of a healthy pregnancy is the production of the hormone progesterone and therefore the activation of Progesterone Induced Blocking Factor (PIBF). With lower levels of progesterone and PIBF in preeclamptic patients comes other risk factors that makes it difficult to have a full-term pregnancy. A person with preeclampsia shows symptoms of chronic inflammation, high blood pressure, mitochondrial dysfunction, and endothelial dysfunction. This consistent rise in blood pressure increases the risk of stroke, liver or kidney rupture and other organ damage for the mother, making it a necessity to deliver the baby early.

According to the data shown, infusing sFlt-1 into pregnant animals stimulates many characteristics of preeclampsia. Because the only management option for preeclampsia is an intravenous injection of magnesium sulfate, which does not treat these symptoms, it is essential to find a treatment to increase vasodilatory factors and decrease vasoactive factors and thus hypertension for the mother. The only treatment available is early delivery of the fetus.

In order to increase vasodilatory factors such as nitric oxide and decrease vasoactive factors such as endothelin-1, 17-orthohydroxyprogesterone caproate, a synthetic progesterone, was used to mimic the effects of natural progesterone. Moreover, we hypothesized that the administration of Progesterone Induced Blocking Factor, a protein resulting from progesterone activating the progesterone receptor, would improve circulating factors such as nitric oxide and endothelin and therefore improve renal and placental mitochondrial function. Taken together, these factors would decrease maternal mean arterial blood pressure thereby allowing the pregnancy to continue.

According to the data collected, this study demonstrates that 17-OHPC improves hypertension, circulating nitrate-nitrite, and endothelin-1. In addition, PIBF, a product of progesterone signaling, safely decreases levels of endothelin-1, blood pressure, and placental mitochondrial reactive oxygen species. Thus, PIBF may be the mechanism whereby 17-OHPC could be beneficial to add to the current management of preeclampsia.

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