Tumor Necrosis Factor Alpha (TNF-α) Blockade improves Natural Killer Cell (NK) Activation, Hypertension, and Mitochondrial Oxidative Stress in a Preclinical Rat Model of Preeclampsia

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Abstract

Preeclampsia (PE) is associated with placental ischemia, hypertension (MAP), increased cytolytic natural killer (cNK) cells, tumor necrosis factor alpha (TNF-α) and mitochondrial Reactive Oxygen Species (mt ROS). The reduced uterine perfusion pressure (RUPP) rat model of PE, exhibits many characteristics of PE. We have shown that blockade of TNF-α, via etanercept (ETAN), decreases MAP, however, the effect of ETAN on cNK cells and mt ROS is unknown. We hypothesized that ETAN would decrease cNK cell and mt ROS in RUPP rats. Rats were divided into 3 groups: normal pregnant (NP) (n=20), RUPP (n=20), RUPP + ETAN (0.4 mg/kg) (n=20). On gestational day 14, RUPP surgery was performed, and ETAN was administered on day 18, MAP, blood and tissue mitochondria were collected on day 19. MAP was elevated in RUPP vs. NP (118 ± 2 vs. 102 ± 1 mmHg, $p<0.05$) which was reduced to 110 ± 2 in RUPP + ETAN ($p<0.05$). cNK cells were increased in circulation (5.9 ± 1.8 vs. 4.0 ± 1.4 % gated cells), placenta (4.8 ± 1.3 vs. 1.9 ± 0.7 %;), and kidney (3.1 ± 0.5 vs. 0.8 ± 0.5% gated cells; of RUPP vs. NP ($p<0.05$) and were lowered to 1.5 ± 0.5%, 1.7 ± 0.7%, 0.8 ± 0.5% gated cells in RUPP + ETAN ($p<0.05$). Placental and renal mt ROS was reduced with ETAN treatment. TNF-α blockade lowered cNK cells, and mt ROS in placental ischemic rats, which could have contributed to lowered blood pressure observed in response to placental ischemia.

Keywords

Hypertension; inflammation and Oxidative Stress

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No Conflicts of Interest

Introduction

Women with preeclampsia have a 2–3-fold increase in plasma TNF-α concentrations compared to normal pregnant women.(1, 2) Serum and placental TNF-α levels are also elevated in rodent models of preeclampsia, such as in the reduced utero placental perfusion model (RUPP) in rats.(3–8) TNF-α is a potent pro-inflammatory cytokine secreted by a variety of cells during an inflammatory response.(1, 8) TNF-α can be secreted by vascular endothelial cells, smooth muscle cells, trophoblasts cells, and epithelial cells.(1, 9, 10) Specifically, in the kidneys, TNF-α can be derived from podocytes, mesangial cells, and epithelial cells of the proximal tubule, thick ascending limb, and collecting duct. TNF-α acts in an autocrine fashion on many immune cells that cause activation of downstream immune and cell signaling events.(10) Multiple studies suggest that TNF-α plays a causative role in hypertension by exerting effects to promote vasoconstriction, endothelial dysfunction, alterations in renal hemodynamics, renal damage, and cardiac injury.(1, 2, 9–13)

Earlier studies by LaMarca *et al.* and others showed that infusion of TNF-α (at 50ng/day) during the last five days of pregnancy in rats increased their blood pressure.(3–7, 14) Furthermore TNF-α administered during late pregnancy increased other plasma markers associated with preeclampsia, such as endothelin-1, soluble fms-like tyrosine kinase (sFlt-1), soluble endoglin (sEng), and ANG II type I receptor autoantibodies (AT1-AAs).(4, 6, 7, 15)

Anti-TNF-α agents are now widely available for the treatment of autoimmune diseases such as rheumatoid arthritis, psoriatic arthritis, juvenile idiopathic arthritis, ankylosing spondylitis, and inflammatory bowel disease.(16) Etanercept (ETAN) binds to the TNF- α and blocks its interaction with the TNF-α cell surface receptors. It is a monoclonal antibody and has a large molecular weight of approximately 150kDa, and is therefore, not expected to cross the human placenta. One study compared the use of three different types of TNF-α inhibition and its association with development of congenital fetal anomalies, it was noted that there was no significant difference between the three groups exposed to anti-TNF-α medications during the first trimester (17).

We have previously shown that blockade of TNF-α by ETAN reduced circulating TNF-α levels and blood pressure by ~15mmHg in RUPP rats.(14) Furthermore these studies showed that HUVEC cells incubated with RUPP serum plus ETAN reduced endothelin-1 (a potent vasoconstrictor), suggesting that TNF-α secretion during preeclampsia is a major contributor to the vasoconstrictor responses that occurs in women with preeclampsia.(14) However, TNF-α blockade did not decrease ET-1 production in the kidney or the placenta of RUPP rats leaving the anti-hypertensive effect during pregnancy unclear. Moreover, it remains unknown what effect ETAN may have on AT1-AA production, however 24 hours post administration is likely not long enough to effect AT1-AA levels in the RUPP rat. A follow up study showed that TNF-α blockade in RUPP rats prevented the enlargement of cardiomyocytes, fibrosis, apoptosis, lowered the expression of mRNA for brain natriuretic peptide (a marker of cardiac hypertrophy), and decreased sFlt-1 amounts in RUPP rats.(11, 15).

It is important to note that preeclampsia is also associated with increased NK cell activation and mitochondrial reactive oxygen species (mt ROS) generation. (8, 18–20) Moreover, we have also demonstrated that RUPP rats with depleted NK cells have decreased serum and placental levels of TNF-α, supporting a role for NK cells to secrete TNF-α, indicating an autocrine effect between TNF-α and NK cells. Our lab has shown through numerous publications the important role of NK cell activation and mt ROS in the pathophysiology of preeclampsia, however little is known about the role of TNF-α in NK cell activation and mt ROS. (18, 19, 21) In summary, all these findings suggest and reinforce that TNF-α contributes to increases in antiangiogenic factors, endothelial dysfunction, oxidative stress and hypertension during pregnancy. Thus signifying that blockade of TNF-α could be an important pathway to target for future therapeutics for this disease. Therefore, the goal of this study was to determine if TNF-α blockade, via ETAN in placental ischemic (RUPP) rats will improve NK cell activation and renal or placental mt ROS.

Methods

The techniques and data that support the findings of this study are available from the corresponding author upon reasonable request.

Timed-pregnant Sprague Dawley (SD) rats purchased from Envigo (Indianapolis, IN) were used in this study. Rats were housed in a 12-hour light and dark cycle each day with temperature-controlled rooms (75°F), in which they were maintained on a normal diet with free access to food and water. All experiments performed were in accordance with the National Institutes of Health guidelines for use and care of animals and all animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Mississippi Medical Center.

Effects of TNF-α blockade on blood pressure in RUPP rats

On gestational day 14 of pregnancy, the reduced uterine perfusion pressure (RUPP) surgery was performed on rats as previously described.(22, 23) Briefly surgical clips were placed over the abdominal aorta and ovarian arteries on the left and right side to reduce blood flow by ~ 40 %.(22, 23) This surgical procedure is often used as a model of preeclampsia in the rat and mimics the pathophysiology of preeclampsia in women.(22, 23) Analgesics were used to provide comfort for the surgical rats, including 5mg/kg carprofen administered via subcutaneous injection, once daily for 2–3 days following RUPP surgical procedure and 0.25% sensor care administered topically after carotid catheter insertion. Pregnant SD rats were randomly divided into 3 groups: Normal Pregnant (NP, n= 20), Reduced Uterine Perfusion Pressure (RUPP, n=20), and RUPP + etanercept (ETAN). ETAN (Enbrel) was administered at a dose of 0.4 mg/kg/day in saline subcutaneously once on gestational day 18. No control saline vehicle was given to RUPP or NP rats, because saline has no physiological effects on rats and surgeries within the control group are unnecessary. This dose of ETAN was used in previous studies and shown to reduce circulating TNF-α levels, blood pressure, and endothelin-1 in RUPP rats.(14) Thus this dose was used in this study to determine its role in NK cell activation and mt ROS generation in RUPP rats. On gestational day 18, under isoflurane anesthesia, catheters were inserted into the carotid artery to

measure the blood pressure. On gestational day 19, conscious blood pressure (MAP), litter size, and pup weights were recorded followed by the collection of blood, placentas, and kidneys for flow cytometry to determine NK cells and mitochondrial isolation for functional assays (24, 25).

The effect of TNF-α blockade on NK cells in RUPP rats

Lymphocytes were isolated from blood placenta, and kidney via centrifugation on a density gradient medium (Lymphoprep, Accurate Chemical & Scientific Corp., Westbury, NY). For flow cytometry, 1×10^6 cells were incubated for 10 minutes at 4 °C with antibodies against rat natural killer cell markers ANK61 and ANK44 as previously described.(26) Cells that stained for ANK61+ and ANK44+ were designated as activated NK cells respectively. The percent of (ANK44+/ANK61+) positive stained cells above the negative control was determined for individual rats and the mean values for each experimental group were calculated.(18)

The effect of TNF-α blockade on mitochondrial oxidative stress (mt ROS) in RUPP rats

Intact placental or renal mitochondria were isolated by differential centrifugation and used to determine mt ROS as previously described.(18) Briefly, once mt were isolated, hydrogen peroxide (H_2O_2) production in placental or renal RUPP mitochondria was determined by fluorescence (555/581 nm excitation/emission for 30 min) using the amplex red reagent (6, 30). All data were normalized per milligram of mitochondrial protein.

Statistical Analysis

All data are presented as mean ± SEM. Data were analyzed by one-way ANOVA with Bonferroni post hoc analysis. Mitochondria reactive oxygen species between RUPP and RUPP+ETAN was analyzed by student *t-test*. All statistical analysis was performed with GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). **P** < 0.05 was considered statistically significant.

Results

Mean arterial pressure (MAP) was elevated in RUPP vs NP (118 ± 2 vs. 102± 1 mmHg, p<0.05) rats as previously shown (Figure 1).(18, 22) MAP decreased in RUPP rats treated with ETAN vs RUPP rats alone (110 ± 2 vs. 118 ± 2 mmHg, p<0.05) (Figure 1). The number of live rat births was reduced for RUPP (8.0±1.0) and RUPP + ETAN (8.0±0.9) rats vs NP rats (14 ± 0.4 pups) (p<0.05) (Figure 2A). Pup weight was decreased in RUPP vs NP rats (2.07±0.05 vs. 2.24 ± 0.05 grams, p<0.05) (Figure 2B) and was not different between RUPP +ETAN vs NP rats (2.15±0.11 vs. 2.24 ± 0.05 grams) (Figure 2B), indicating improvements in pup weights in response to TNF-α inhibition in response to placental ischemia.

Circulating (5.9 ±1.8 vs 4.0±1.4 % gated) (Figure 3A), renal (3.1 ±0.5 vs 0.9±0.3 % gated, p<0.05) (Figure 3B), and placental (4.8 ±1.3 vs 1.9±0.7 % gated, p=0.055) (Figure 3C) activated NK cells are elevated in RUPP vs. NP rats. ETAN drastically decreased the circulating (1.5 ±0.5 vs 5.9±1.8 % gated, p<0.05) (Figure 3A), renal (0.8 ±0.5 vs 3.1±0.5 %

gated, p<0.05) (Figure 3B), and placental (1.7 ±0.7 vs 4.8±1.3 % gated) (Figure 3C) activated NK cells in RUPP rats vs. RUPP. We have previously shown renal and placental respiration and mt ROS to be significantly different in RUPP rats compared to NP rats.(27) Mt ROS in the kidney was reduced by 21% and 29% in placentas in RUPP + ETAN vs RUPP rats; renal-0.79 ±0.02 vs 1.00±0.05 % gated, (p<0.05) (Figure 4A) and placenta0.71±0.06 vs 1.00±0.07 % gated, (p<0.05) (Figure 4B).

Discussion

In our current study we demonstrate that TNF-α blockade with ETAN not only reduces blood pressure, but lowers circulating, renal, and placental activated NK cells, improves fetal weight to a level that is no longer significantly less than normal pregnant pup weights, and decreases placental and renal mitochondrial oxidative stress in the hypertensive pre-clinical rat model of preeclampsia.

Importantly, this study demonstrates that use of ETAN does not worsen the fetal outcomes that have been demonstrated with placental ischemia. This study also shows that treatment with ETAN improved hypertension, suggesting that it could be used as a potential therapeutic to prolong pregnancies complicated by preeclampsia. RUPP rats are shown to have an increase in circulating, placental, and renal activated NK cells (20, 24) however, it had not been previously demonstrated that ETAN treatment decreased activated NK cells in circulation or in tissues. This novel finding demonstrates that ETAN has a positive effect on the immune response during placental ischemia that improved the clinical characteristics observed in preeclampsia.

Another important finding of our study is that ETAN treatment reduced mt ROS in both the placenta and kidney, supporting a link between NK cell activation and TNF-α causing mt oxidative stress. Granzymes A & B, serine proteases, majorly secreted by activated NK cells, have been shown to cause mitochondrial dysfunction and mt ROS in *in vitro* as well as in isolated mitochondria. [28, 29]. In fact, Chiusolo *et. al.*, published that granzyme A and B enter mitochondria via Tob55/Sam50 and Tim22 channels located on the outer and inner mitochondrial membranes and go on to cleave Complex I subunits. This ultimately results in mt dysfunction, mt ROS, and apoptosis.(28) (29) Collectively, these studies demonstrate that the ultimate targets of granzymes are located within the mitochondria and can cause mitochondrial injury, dysfunction, and ROS generation.

We have previously, and were the first to reveal, that RUPP rats exhibit mt dysfunction and mt ROS in both placenta and kidney,.(27) We further went on to demonstrate that NK cell activation is one of the mechanisms behind mt oxidative stress in RUPP rats.(18, 21) However, the downstream signaling pathways of NK cell activation that mediate mt dysfunction in our RUPP rats is still yet to be explored.

ETAN is prescribed to treat autoimmune diseases, such as rheumatoid arthritis, ankylosing spondylitis, psoriasis, psoriatic arthritis and juvenile rheumatoid arthritis, that is safe for women before pregnancy and during pregnancy (30,31). Treatment with ETAN has been shown to reduce activation of NK cell cytoxicity in patients with rheumatoid arthritis or

psoriatic arthritis (RA/PsA)(32). The ability of ETAN to restore NK cell balance has been demonstrated in women with recurrent pregnancy losses (33). In the available literature to date clinical studies indicate no harm to the mother or fetus in women with inflammatory bowel disease taking anti-TNF agents during pregnancy (16,17,30–33). Based on our studies ETAN appears to be a therapeutic target for pre-eclampsia, because it reduces the blood pressure, NK cell activation, which results in improved mt ROS and fetal weight in response to placental ischemia. Furthermore, ETAN during pregnancy has also shown to have no increase in the risk of congenital malformations in infants, one possiblly due to ETAN having the shortest half-life of all TNF-α inhibitors (30). A few studies have shown low levels of ETAN in fetal cord blood and neonatal blood after delivery. It was not present in the fetal blood stream after delivery (31)

In summary ETAN reduced blood pressure, NK cell activation, and mt ROS in placental ischemic rats while improving fetal weight. Thus, ETAN could be used as a potential therapeutic for improving hypertension, inflammation, and mitochondrial oxidative stress during complicated pregnancies.

Acknowledgments

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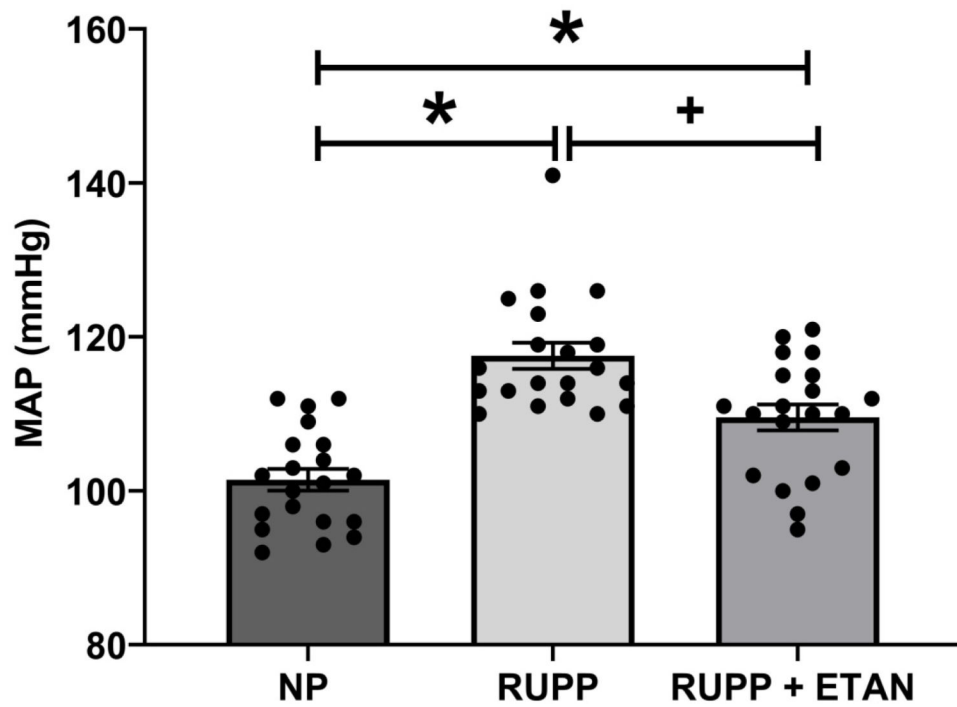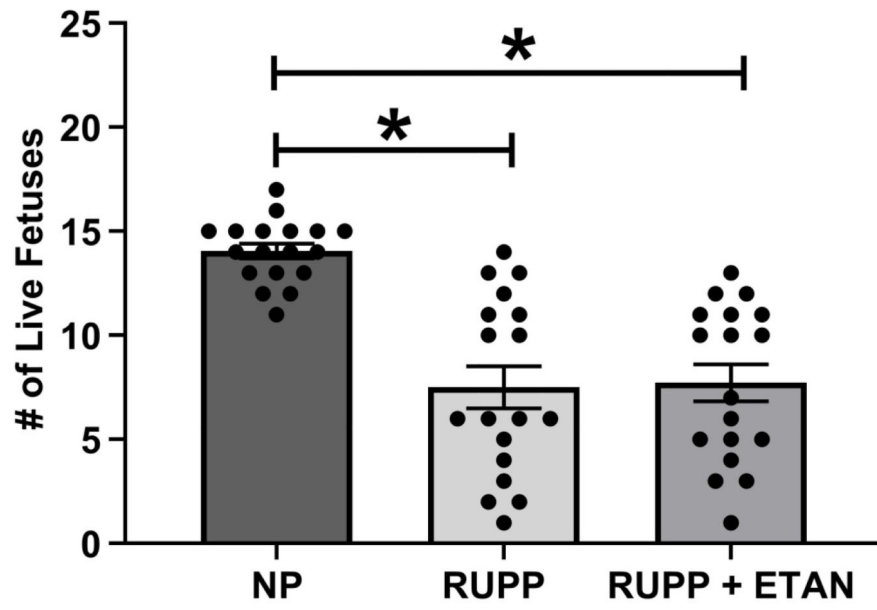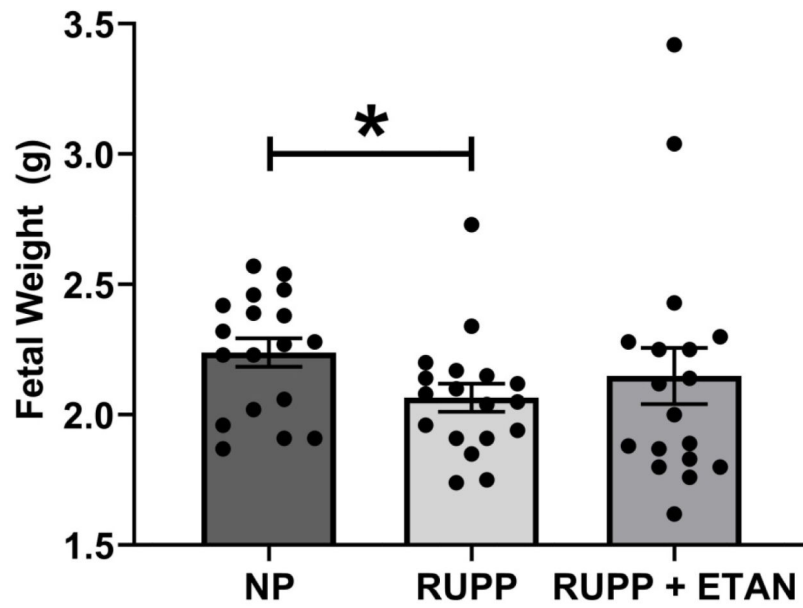
Figure 1.
MAP was measured in RUPP (n=20), normal pregnant (n=20) (NP), and ETAN administered to RUPP (n=20) rats.. Statistical differences were achieved using one-way ANOVA with Bonferroni post hoc analysis, *p < 0.05 vs NP and +p < 0.05 vs. RUPP.
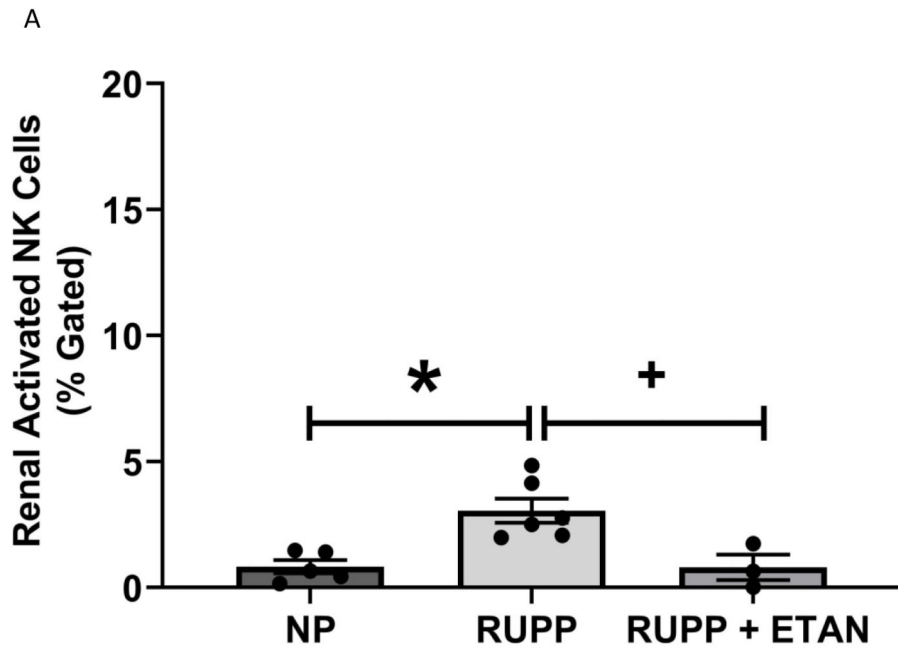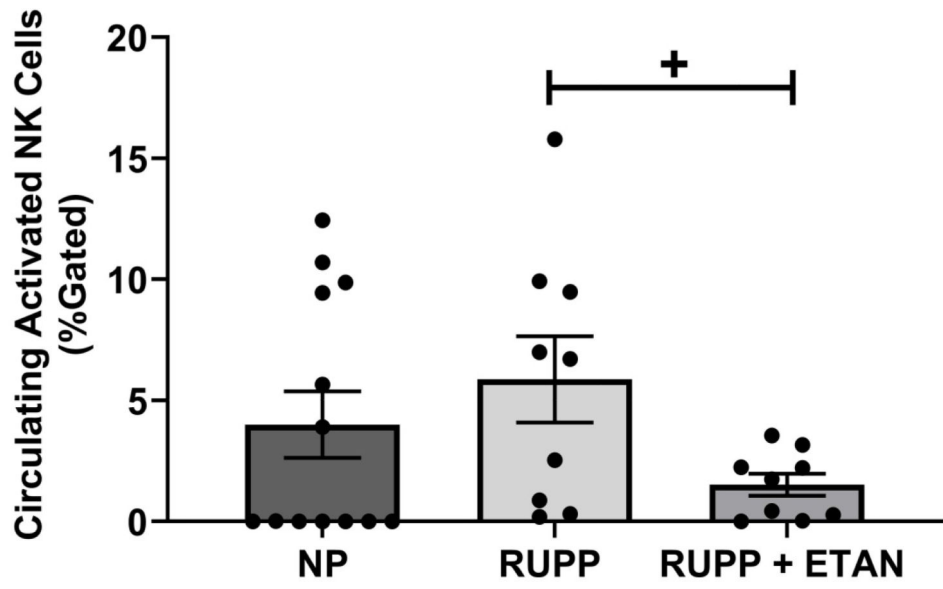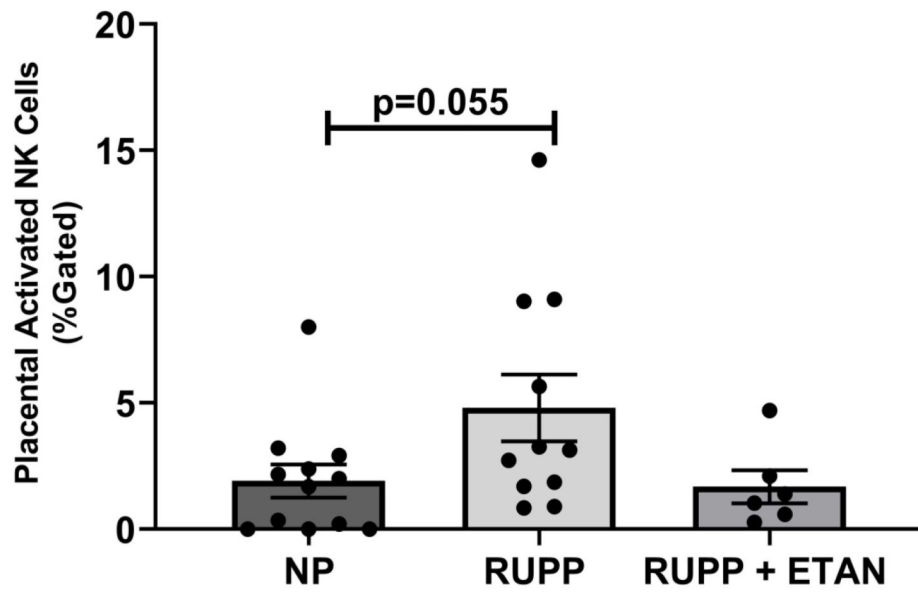
A

B

Figure 2.
A) The number if live pups and **B)** Fetal weight was recorded NP, RUPP, and RUPP + ETAN ratsStatistical differences were achieved using one-way ANOVA with Bonferroni post hoc analysis, *p < 0.05 vs NP.
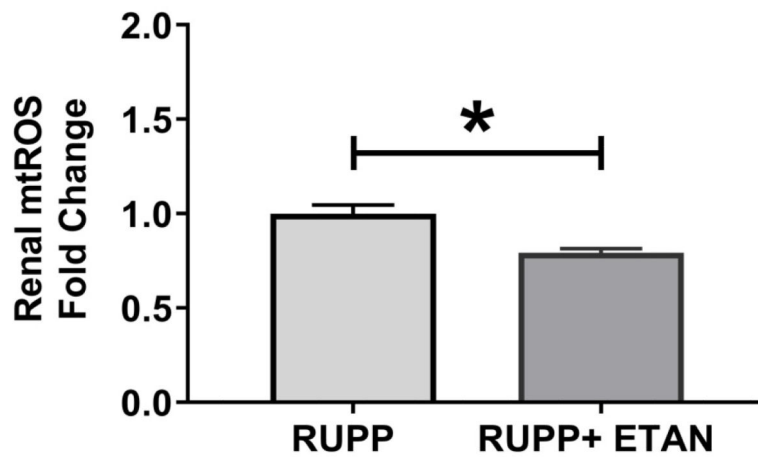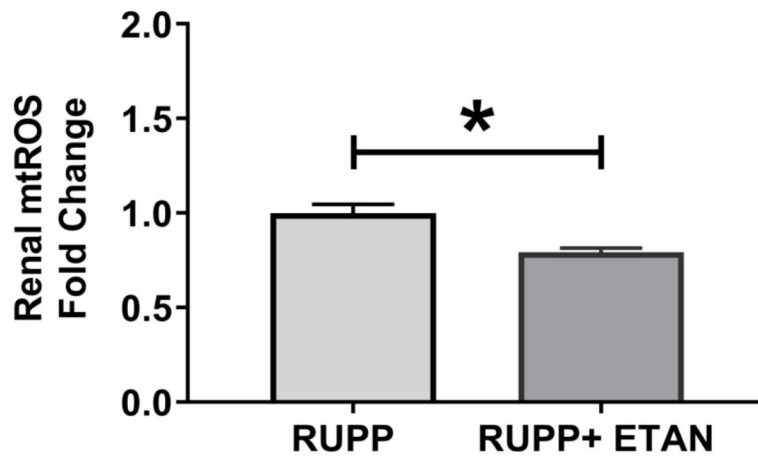
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Figure 3.

A) Circulating NP (n=13), circulating RUPP (n=9), circulating RUPP + ETAN (n=9). **B)** Placental NP (n=5), placental RUPP (n=6), placental RUPP + ETAN (n=3). **C)** Renal NP (n=12), renal RUPP (n=11), renal RUPP + ETAN (n=6). Activated NK cells were quantified using flowcytometry. Statistical differences were achieved using one-way ANOVA with Bonferroni post hoc analysis, *p < 0.05 vs NP and [+]p < 0.05 vs. RUPP.

A

B

Figure 4.
A) Renal and **B)** Placental mitochondrial oxidative stress was measured in RUPP (n=3) and RUPP + ETAN (n=3) rats. Statistical differences were achieved using *student t- test*, *p < 0.05 vs NP.