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American Journal of Physiology Regulatory Integrative and Comparative Physiology
2016 FEB 15 ; 310(4): R346-R354
2015 DEC 16 (first published online)
doi: 10.1152/ajpregu.00388.2015

Publisher: American Physiological Society

Articles in PresS. Am J Physiol Regul Integr Comp Physiol (December 16, 2015). doi:10.1152/ajpregu.00388.2015

Vitamin D supplementation improves pathophysiology in a rat model of preeclampsia

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Running Head: Vit D improves pathophysiology in preeclamptic rat model

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Abstract

Deficiency of Vitamin D (VD) is associated with preeclampsia (PE), a hypertensive disorder of pregnancy characterized by proinflammatory immune activation. We sought to determine if VD supplementation would reduce the pathophysiology and hypertension associated with the Reduced Uterine Perfusion Pressure (RUPP) rat model of PE. Normal pregnant (NP) and RUPP rats were supplemented with VD2 or VD3 (270 IU and 15 IU/day, respectively) on gestation days 14-18 and mean arterial pressures (MAPs) measured on day 19. MAP increased in RUPP to 123±2 mmHg compared to 102±3 mmHg in NP and decreased to 113±3 mmHg with VD2 and 115±3 mmHg with VD3 in RUPP rats. Circulating CD4+ T cells increased in RUPP to 7.90±1.36% lymphocytes compared to 2.04±0.67% in NP but was lowered to 0.90±0.19% with VD2 and 4.26±1.55% with VD3 in RUPP rats. AT1-AA, measured by chronotropic assay, decreased from 19.5±0.4 bpm in RUPPs to 8.3±0.5 bpm with VD2 and 15.4±0.7 bpm with VD3. Renal cortex endothelin-1 (ET-1) expression was increased in RUPP rats (11.6±2.1-fold change from NP) and decreased with both VD2 (3.3±1.1-fold) and VD3 (3.1±0.6-fold) supplementation in RUPP rats. Plasma soluble FMS-like tyrosine kinase-1 (sFlt-1) was also reduced to 74.2±6.6 pg/ml in VD2-treated and 91.0±16.1 pg/ml in VD3-treated RUPP rats compared to 132.7±19.9 pg/ml in RUPP rats. VD treatment reduced CD4+ T cells, AT1-AA, ET-1, sFlt-1, and blood pressure in the RUPP rat model of PE and could be an avenue to improve treatment of hypertension in response to placental ischemia.

Key Words: Hypertension, Immune Activation, Preeclampsia, Vitamin D

Introduction

Preeclampsia (PE) is a clinical condition occurring in up to 7% of pregnancies in the United States commonly manifesting in late-gestation (>20 weeks gestation) with hypertension, placental ischemia and low birthweight (5, 27, 47, 48, 58). Current treatment strategies for preeclampsia are targeted at safely lowering blood pressure and alleviating maternal complications (5, 48).

PE pregnancies are characterized by an abnormal immune profile compared to that seen in normal pregnancies. PE women exhibit an altered immune balance favoring proinflammatory factors such as CD4+ T cells, B cells, inflammatory cytokines and autoantibodies to the angiotensin type I receptor (AT1-AA) which are known to stimulate production of anti-angiogenic protein soluble FMS-like tyrosine kinase-1 (sFlt-1) (18, 33, 34, 56, 57). In contrast, anti-inflammatory T regulatory cells (TREGs) are decreased in PE (22, 53, 56). These immune alterations are recapitulated in the established experimental model of PE, the Reduced Uterine Perfusion Pressure (RUPP) rat (1, 20, 21). Adoptive transfer of CD4+ T cells from RUPP rats induces hypertension, AT1-AA, inflammatory cytokines and sFlt-1, and ET-1 in normal pregnant rats, indicating the significant role these cells play in the pathogenesis of this disease (63). Furthermore, AT1-AA and sFlt-1 play a significant role in the development of endothelial dysfunction and hypertension in PE and have been found to correlate with PE severity in patients (15, 25, 30, 43, 60, 64, 65, 67, 69, 71, 72). AT1-AA infusion induces

many pathophysiological characteristics of PE including increased blood pressure, vascular resistance, ET-1, and sFlt-1 (8, 35). Although the contribution of immune factors in the pathogenesis of preeclampsia is well established, immune therapy in preeclamptic women is limited by the potential for teratogenic effects of many anti-inflammatory and anti-hypertensive drugs.

The steroid hormone Vitamin D (VD) is well established as a necessary factor for healthy calcium homeostasis, however, emerging findings of nonclassical effects of VD signaling have encouraged studies examining its potential in many disease states. VD has recently been recognized for its role as a potent factor in immune regulation in human physiology (6, 29, 31, 41, 44). Vitamin D receptor activation on immune cells inhibits proliferation of CD4+ T cells, B cell activation and also increases transcription of FoxP3+ T regulatory cells (TREGs) (10, 29, 31, 39). Studies in clinical populations vary with regard to the potential benefit of the anti-inflammatory effects of VD in disorders in which immune activity is known to play a role, such as hypertension and PE. However, it has been suggested that VD deficiency (<50 nmol/l) in both mid-term and late-term gestation is associated with PE in pregnant women (4, 7, 66). Importantly, VD supplementation has been shown to reduce incidences of PE and improve fetal growth in some clinical studies, however, there remains a need for large-scale, standardized clinical trials to confirm these findings (23, 24, 28). There is little experimental data investigating the role of VD in placental ischemia and the immunoregulatory

effects of VD in rodent models of PE have not been fully evaluated. In order to examine this, we utilized both forms of VD that are metabolized in humans and animals, Vitamin D2 (VD2) and Vitamin D3 (VD3), for supplementation to the RUPP rat model of PE. We recently demonstrated that supplementation of VD2 or VD3 decreased circulating CD4+ T cells and lowered blood pressure in the RUPP rat model of PE (14). However, hypertensive mechanisms associated with T cell activation, such as AT1-AA, ET-1, inflammatory cytokines and sFlt-1 in response to placental ischemia were not determined. Moreover, the effect of Vitamin D supplementation on fetal growth and survival were not examined. Therefore, we hypothesized that Vitamin D administration to the RUPP rat model of placental ischemia would reduce inflammatory T cells, leading to a decrease in AT1-AA, ET-1, sFlt-1 and ultimately blood pressure during pregnancy.

Materials and Methods

All procedures involving animals in this study were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. Animal experiments were conducted on timed-pregnant Sprague Dawley rats (Harlan, Indianapolis, IN) that were housed under a 12 hour light/dark cycle and fed standard laboratory chow diet.

Vitamin D administration to RUPP rats

For our studies we used an established model of placental ischemia, the Reduced Uterine Perfusion Pressure (RUPP) rat. The RUPP procedure in Sprague-Dawley rats has been shown to induce many of the pathological characteristics of preeclampsia (1, 20, 21). Six groups of rats were utilized for this study: Normal Pregnant (NP) (N=15), Normal Pregnant + Vitamin D2 (NP+VD2) (N=6) (County Line Pharmaceuticals, Brookfield, WI), Normal Pregnant + Vitamin D3 (NP+VD3) (N=6) (Enfamil, Glenview, IL), RUPP (N=19), RUPP + Vitamin D2 (RUPP+VD2) (N=11) and RUPP + Vitamin D3 (RUPP+VD3) (N=13). The RUPP procedure was performed in pregnant rats under isofluorane anesthesia on gestational day 14 (GD14) by placing a constrictive silver clip on the abdominal aorta superior to the bifurcation (0.203 mm) and on both bilateral uterine arteries at the ovarian end (0.100 mm), as described previously (1, 20, 21). VD2 (ergocalciferol) and VD3 (cholecalciferol) were administered to NP and RUPP rats on GD14-18 at a dose of 270 IU and 15 IU, respectively, by daily gavage. Doses were determined based on a concentration:effect experiment our laboratory previously performed to determine the minimal dose that had an effect on blood pressure and T cells in RUPP rats. On GD18, indwelling carotid catheters were inserted and on GD19 blood pressure was assessed consciously via pressure transducer (Cobe II Transducer CDX Sema, Birmingham, AL) followed by sacrifice and collection of whole blood and tissues and weighing of pups and placentas.

Determination of CD4+ T cells, CD45+ B cells and FoxP3+CD25+ T regulatory cells (TREG) by Flow cytometry

Flow cytometry was used to assess the effect of Vitamin D on differentiation of immune lymphocytes. Whole blood was diluted with RPMI 1640 (Invitrogen, Grand Island, NY) and layered over Ficoll-Hypaque gradient with Lymphoprep® commercially available reagent (Accurate Chemical Corp, Westbury, NY). The isolated lymphocytes were extracted and centrifuged. The lymphocytes were then blocked in mouse and goat serum blocking buffer and washed with an RPMI 1640/FBS/EDTA solution. Lymphocytes were incubated at 4°C with antibodies for CD4, CD45R, CD25 (BD Biosciences, San Jose, CA) and FoxP3 (R and D, Kingstown, RI). Cells were then washed and incubated with fluorescent secondary antibodies APC (BD Biosciences, San Jose, CA), PE and FITC (Southern Biotech, Birmingham, AL) and analyzed for expression of CD4, CD45, FoxP3 and CD25 via Gallios® flow cytometer (Beckman Coulter, Indianapolis, IN). The resulting data was gated and analyzed for populations of CD4+ (T cells), CD45R+ (B cells) and CD4+/CD25+/FoxP3+ (TREG cells) with Kaluza® software (Beckman Coulter, Indianapolis, IN).

Determination of AT1-AA

The effect of Vitamin D on AT1-AA in RUPP rats was quantified using the rat neonatal cardiomyocyte assay as previously described (16, 17, 64). Briefly, AT1-AA was isolated by epitope binding and column purification from total IgG and chronotropic responses were measured and expressed as beats per minute (bpm).

Analysis of Renal Cortex Preproendothelin-1 Expression

Tissue preproendothelin-1 (PPET) levels were measured in homogenized renal cortex by real time PCR (qRT-PCR). Total RNA was isolated with the RNeasy Protect Mini Kit (Qiagen, Germantown, MD) performed according to the manufacturer provided instructions. cDNA was generated from 1 µg total RNA with an iScript cDNA Synthesis Kit (BioRad, Hercules, CA). qRT-PCR was performed using iQ SYBR Green Supermix (BioRad, Hercules, CA) and fluorescence detected on a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA). Life technologies provided primer sequences that were used for PPET measurement in this study as has been previously described (62):

Forward: ctaggtctaagcgatccttg, Reverse: tctttgtctgcttggc. Levels of mRNA were calculated using the mathematical formula for $2^{-\Delta\Delta Ct}$ ($2^{\text{avg. Ct gene of interest} - \text{avg Ct beta actin}}$) which has been previously recommended by Applied Biosystems (Applied Biosystems User Bulletin, No. 2, 1997).

Measurement of Circulating 25(OH) VD

Plasma isolated by centrifugation of whole blood on day of sacrifice was analyzed for 25(OH) VD via LC/MS analysis. 10ul of 0.2ng/ul 25(OH) Vitamin D3 internal control was added to 200ul rat plasma followed by acetonitrile (500ul). Samples were then centrifuged at 10,000xG and the organic phase extracted from solution by drying with nitrogen gas. Samples were reconstituted with water and re-extracted with solid phase extraction (SPE) column (Waters Corp, Milford MA), washed with methanol and eluted with ethyl acetate prior to analysis**.** All samples were analyzed utilizing an autosampler on a Dionex Ultimate 3000 High-Performance Liquid Chromatography system (Dionex, Banmockburn, IL) prior to analysis on an ABsciex 4000 Q trap tandem mass spectrometer with electrospray ionization (ABsciex, Foster City, CA).

Determination of Circulating sFlt-1, Nitrate/Nitrite, TNF-α and IL-6

Commercially available Enzyme-linked Immunosorbant Assay (ELISA) from R & D systems were utilized to measure sFlt-1, TNF-α and IL-6 in rat plasma (Minneapolis, MN). ELISA to measure plasma nitrate/nitrite for determination of circulating nitric oxide was obtained from Cayman Chemicals (Ann Arbor, MI).

Statistical analysis

All data were expressed as mean +/- standard error of the mean. Statistical analysis was performed in GraphPad Prism® (La Jolla, CA) software utilizing

standard Student's t-test and/or one-way ANOVA comparing the control and treated groups. P value <0.05 was considered significant.

Results

VD treatment improved blood pressure in RUPP rats

Mean arterial pressures (MAPs) in NP rats was 102.2±3.2 mmHg and was not significantly changed in NP+VD2 (92.5±4.4 mmHg, P=0.11) or NP+VD3 rats (93.3±4.4 mmHg, P=0.14) [Figure 1a]. MAP increased significantly to 122.5±2.0 mmHg (*P<0.0001) in RUPP rats compared to NP rats. VD2 and VD3 treatment significantly reduced MAP in RUPP rats to 113.4±3.4 (*P<0.05) and 115.4±2.7 mmHg (*P<0.05), respectively.

VD reduced fetal death in RUPP rats and did not cause adverse fetal effects in NP rats

Intrauterine growth restriction as measured by average pup weight on GD19 decreased to 1.84±0.05 g (*P<0.01) in RUPP rats compared to 2.28±0.12 g in NP and was unaltered in RUPP+VD2 (1.95±0.09 g) or RUPP+VD3 (1.89±0.10 g) groups [Figure 1b]. In addition, we observed no differences in pup weight in NP+VD2 (2.37±0.05 g) or NP+VD3 (2.20±0.10 g) rats compared to NP, indicating that VD did not adversely affect pup growth. Reabsorptions were found much

more frequently in RUPP rats than NP (4.62±1.29 vs 0.07±0.02 reabsorbed/live pups, respectively, *P<0.001) [Figure 1c]. NP rats treated with VD2 or VD3 did not have altered reabsorption rates compared to NP (0.06±0.05 vs 0.06±0.03 reabsorbed/live pups, respectively). Importantly, VD2 treatment reduced fetal death to 1.57±0.57 reabsorbed/live pups (*P=0.05) and VD3 treatment to 1.79±0.46 reabsorbed/live pups (*P<0.05) in RUPP rats, demonstrating that VD treatment was able to improve fetal survival in the presence of placental ischemia. Placental weights did not change from NP rats (0.62±0.05 g) in either NP+VD2 (0.58±0.01 g) or NP+VD3 (0.54±0.02 g). RUPP rats had significantly reduced placental weight (0.50±0.03 g, *P<0.05) compared to NP [Figure 1d]. Neither VD2 (0.51±0.03 g) nor VD3 (0.54±0.03 g) supplementation in RUPP rats altered placental weight. Placental efficiency as defined by placenta/fetal weight ratio was not altered in RUPP rats (0.27±0.01) compared to NP (0.28±0.02) [Figure 1e]. NP+VD2 (0.24±0.01) nor NP+VD3 rats (0.25±0.01) had altered placental efficiency compared to NP rats and VD2 (0.26±0.01) and VD3 administration (0.29±0.02) to RUPP rats did not alter this ratio either.

Circulating 25(OH) VD was not altered in RUPP rats or with VD treatment

RUPP rats have increased circulating 25(OH) VD compared to NP, indicating that placental ischemia did not induce VD deficiency in mid to late gestation (data not shown). Neither VD2 nor VD3 increased circulating VD levels in RUPP rats.

CD4+ T cells were decreased in RUPP rats treated with VD

CD4+ T cells were assessed and analyzed as a percentage of total whole blood lymphocytes. Circulating CD4+ T cells were increased to 7.90±1.36% lymphocytes (*P<0.01) in RUPP rats compared to 2.04±0.67% lymphocytes in NP [Figure 2a]. We observed a decrease in CD4+ T cell population to 0.90±0.19% lymphocytes (*P<0.05) in RUPP+VD2 and a modest decrease in RUPP+VD3 to 4.26±1.55% lymphocytes (P=0.14). CD4+ T cells were increased in our NP rat groups treated with VD compared to untreated NP, 4.52±1.86% lymphocytes in NP+VD2 (P=0.15) and 10.23±6.44% lymphocytes in NP+VD3 (*P<0.05).

CD4+/CD25+/FoxP3+ TREGs were decreased in RUPP rats and unaltered by VD

TREGs as assessed by CD4+/CD25+ and intracellular FoxP3+ were assessed as percentage of CD4+/CD25+ lymphocytes. VD treatment increased circulating TREG populations from 1.87±0.10% CD4+/CD25+ lymphocytes in NP rats to 4.58±2.04% CD4+/CD25+ lymphocytes in NP+VD2 (P=0.17) and 9.57±6.76% CD4+/CD25+ lymphocytes in NP+VD3 (P=0.23) [Figure 2b]. This increase in TREGs indicates that the increase in total CD4+ T cells seen in these groups may be the result of increased FoxP3+ TREG cell differentiation. RUPP rats had significantly less TREGs at 0.65±0.42% CD4+/CD25+ lymphocytes (*P=0.05) compared to NP rats. In the presence of placental ischemia VD modestly

increased TREGS to 1.76±1.03% CD4+/CD25+ lymphocytes (P=0.26) in RUPP+VD2 rats and 4.54±2.82% CD4+/CD25+ lymphocytes (P=0.086) in RUPP+VD3 rats, although these changes did not reach significance.

Proinflammatory cytokines were reduced with Vitamin D treatment in RUPP rats

We assessed circulating TNF-α and IL-6 levels in our RUPP rats treated with VD. Although TNF-α increased 5 fold in RUPP rats (103.5±38.05 pg/ml) compared to NP rats (22.7±9.2 pg/ml, P=0.07), the variation in the RUPP rat group was greater than observed in previous studies, and therefore did not reach statistical significance [Figure 3a]. However a lowering of TNF-α levels in RUPP+VD2 (12.6±5.3 pg/ml, P=0.09) and RUPP+VD3 (52.7±25.7 pg/ml, P=0.29) was observed. Circulating IL-6 levels were significantly increased in RUPP rats (253.3±60.6 pg/ml, *P<0.05) compared to NP (93.3±15.1 pg/ml) [Figure 3b]. Importantly, VD2- (62.6±11.4 pg/ml, *P<0.05) and VD3- (98.2±17.5 pg/ml, *P=0,05) treated RUPP rats had significantly lower plasma IL-6 levels compared to untreated RUPP rats.

AT1-AA levels were decreased in RUPP rats treated with VD

Serum levels of AT1-AA were significantly decreased in RUPP+VD2 rats to 8.3±0.5 beats/min (*P<0.0001) and in RUPP+VD3 rats to 15.4±0.7 beats/min (*P=0.001) compared to untreated RUPP rats (19.5±0.4 beats/min) [Figure 4a].

B cell populations were increased in RUPP rats and unaltered with VD treatment

B cell populations were assessed by flow cytometry and expressed as a percentage of total lymphocytes that stained positive for CD45R. VD2 and VD3 treatment increased B cells to 9.73±4.85% lymphocytes (*P<0.05) and 8.11±4.30% lymphocytes (P=0.06) compared to NP rats (2.95±0.76% lymphocytes) [Figure 4b]. RUPP rats exhibited increased B cells compared to NP rats (11.09±3.12% lymphocytes, *P=0.05). VD2 and VD3 treatment did not significantly change B cells from RUPP rats with 6.28±1.71% lymphocytes in RUPP+VD2 and 5.27±1.46% lymphocytes in RUPP+VD3. These data indicate that B cells were not changed with VD treatment in the presence of placental ischemia, however their secretion of AT1-AA was decreased.

sFlt-1 plasma levels decreased with VD treatment in RUPP rats

Plasma sFlt-1 levels were assessed with ELISA assay. sFlt-1 levels were significantly increased in RUPP rats (132.7±19.9 pg/ml, *P<0.05) compared to NP rats (42.5±8.1 pg/ml) [Figure 5]. VD2 treatment significantly reduced sFlt-1 to (74.2±6.7 pg/ml, *P<0.05) in RUPP rats and VD3 reduced levels to (91.0±16.1 pg/ml, P=0.15), although this did not reach significance.

Renal cortex preproendothelin-1 expression was decreased with VD treatment in RUPP rats while nitric oxide levels were unchanged

Plasma nitric oxide (NO) levels assessed as nitrate/nitrite were not changed with VD2 (78.6±23.5 uM) or VD3 treatment (115.3±19.1 uM) in RUPP rats compared to untreated RUPPs (89.5±9.1 uM) [Figure 6a]. However, vasoconstrictor, endothelin-1 (ET-1) increased in RUPP rats, and was significantly lowered when treated with VD. Preproendothelin-1 (PPET) mRNA expression was analyzed as fold change with NP normalized to 1. PPET was significantly increased in RUPP rats (11.6±2.1-fold change, *P<0.05) compared to NP rats (1.0±0.9) [Figure 6b]. VD2 and VD3 treatment in RUPP rats reduced PPET levels to 3.3±1.1-fold change (*P<0.05) and 3.1±0.6-fold change, respectively (*P<0.05).

Discussion

In this study we present evidence that Vitamin D (VD) supplementation reduces immune pathogenesis and improves blood pressure and fetal survival in response to placental ischemia-induced hypertension during pregnancy. At present, safe therapeutics for immune activation in PE patients are restricted by potential teratogenic effects of immunosuppressive drugs. While PE has a complex etiology that at this time is not fully elucidated, immune mechanisms are suggested to play a significant role in the currently accepted two-stage theory of the pathogenesis of PE (54). VD is a safe supplement in pregnant women with no known adverse effects to either mother or neonate and may reduce the incidence of PE and improve fetal growth (23, 24, 28, 55).

Insufficient placental perfusion in the RUPP model induces a cascade of events including immune activation, AT1-AA, ET-1 and sFlt-1 production ultimately leading to hypertension and decreased fetal weight (1, 20, 21). The potential of Vitamin D supplementation to reduce immune activation and other pathological factors that are associated with placental ischemia has not been previously evaluated prior to the current study. Therefore, we sought to test the hypothesis that VD improves immune activation, production of AT1-AA, ET-1, sFlt-1, and subsequently, blood pressure in the RUPP model of PE. Furthermore, we administered VD to NP rats to evaluate potential adverse fetal effects with VD administration during pregnancy.

Many proinflammatory cell types, including CD4+ cells, natural killer (NK) cells and TH17 cells, have been found to play a role in the pathogenesis of placental ischemia. In particular, the role of CD4+ T cells has been well established. Adoptive transfer studies have confirmed that CD4+ T cell function in RUPP rats is altered to promote the production of AT-AA, ET-1 and sFlt-1 in otherwise healthy pregnant rats as mechanisms of increasing blood pressure during pregnancy (12, 49, 50). CD4+ T cell population was increased in RUPP compared to NP rats and TREGs were decreased, consistent with what has been published previously (3, 11, 14, 49, 63). Data in the literature has shown that VD reduces proinflammatory CD4+ T cells and increases proliferation of TREGs in

non-pregnant animals (10, 29, 31, 39). As predicted by these previous findings, VD treatment to NP rats did increase both CD4+ T cells and TREGs. In this report we recapitulate a previous study in that VD2 and VD3 supplementation in RUPP rats reduced total CD4+ T cells (14). Although we did see a decrease in the total CD4+ T cell number there was no increase in the Treg subpopulation of CD4+T cells with VD treatment in RUPP rats. Therefore, VD was ineffective in the presence of placental ischemia-associated CD4+ T cell dysregulation but under normal conditions was able to sitmulate the percentage of CD4+ Treg cells in NP rats, which did not lead to adverse effects in the mother or fetus. The effect of VD treatment in the proliferation of other proinflammatory cell types known to contribute to the pathogenesis of PE, such as NK and TH17 cells, was not evaluated in this study and may be assessed in future experiments. A classical marker of proinflammatory T cell activation is the production of inflammatory cytokines. We found that VD supplementation into RUPP rats significantly reduced IL-6 levels and lowered TNF-α. Our lab has shown that TNF-α or IL-6 infusion into pregnant rats induces AT1-AA and sFlt-1 production (36, 38, 51). Therefore, VD treatment in rats with placental ischemia altered activation of CD4+ T cells thereby possibly causing a reduction in proinflammatory cytokines, and a decrease in both AT1-AA and sFlt-1.

Many studies have confirmed that AT1-AA and sFlt-1 are central mediators of hypertension in PE women (15, 43, 60, 64). Clinical data has confirmed that

severe PE in patients is associated with higher circulating AT1-AA and sFlt-1 levels, demonstrating a link between these mediators and pathogenesis of the disease (59, 60). This study is the first to measure changes in the production of AT1-AA and sFlt-1 in response to VD supplementation. AT1-AA production is a fairly unique factor to PE patients and is not found in measurable levels in normal pregnancy. In the absence of placental ischemia, AT1-AA infusion into pregnant rats induces hypertension, endothelial dysfunction and sFlt-1 production (8, 51).The cascade of pathological events in PE that induce AT1-AA remain under investigation, however, it is known that AT1-AA are induced by CD4+ T cells derived from RUPP rats and that they are produced by B cells (37, 50). Therefore, we evaluated B cell populations in response to VD supplementation in NP and RUPP rats and found that B cells were not significantly reduced. However, B cell production of AT1-AA was reduced in RUPP rats with VD2 and VD3, which was also associated with decreases in plasma sFlt-1 levels. sFlt-1 is an anti-angiogenic peptide that acts as a soluble scavenger sequestering vascular endothelial growth factor (VEGF). sFlt-1 infusion into pregnant rats increases blood pressure and intrauterine growth restriction (9, 45). Importantly, there is a strong mechanistic tie between AT1-AA and sFlt-1 as studies have confirmed that AT1-AA stimulates sFlt-1 production, which is associated with reduction of blood pressure in pregnant rats (8, 51, 71). In this study, we observed a similar link as decreased AT1-AA in RUPP rats were parallel with

sFlt-1 reduction. Therefore, VD reduction of AT1-AA likely led to a decrease in sFlt-1 production and ultimately, a reduction in blood pressure.

VD has been shown to improve endothelial dysfunction and VDR signaling in vascular cells (46, 68). Endothelial dysfunction is an observed pathological event in the RUPP model, evident by vascular impairment, reductions in nitric oxide (NO) bioavailability and production of endothelin-1 (ET-1) (3, 13, 32, 37, 40). Interestingly, we did not observe a change in circulating nitric oxide (NO) levels in RUPP rats treated with VD. ET-1 is a potent vasoconstrictor and is emerging as an important player in the vascular dysfunction observed in PE (19). VD supplementation significantly reduced renal production of ET-1 in RUPP rats in correlation with reduction of AT1-AA. ET-1 has been shown to mediate, at least in part, the hypertensive responses of RUPP and AT1-AA-infused, Sflt-1 treated and to RUPP CD4+ T cells in pregnant rats, (19, 52, 62). The observed improvement of endothelin-1 levels suggest improved function and endothelial activation with VD supplementation was likely a result of reduced CD4+ T cells, sFlt-1 secretion and AT1-AA-mediated signaling, all of which could play a role in the observed reduction of blood pressure.

A significant concern in clinical studies of PE therapeutics is that maternal symptoms of PE are to be improved without causing adverse effects on the

442 growing fetus. NP rats treated with VD had a slight decrease in blood pressure,
443 although this did not reach significance. However, we have demonstrated that
444 VD administration reduced blood pressure and fetal death in RUPP rats but did
445 not negatively affect fetal weight, demise or placental efficiency in NP or RUPP
446 rats. Therefore, the present study suggests that VD supplementation in NP rats is
447 not associated with reduced placental blood flow or fetal growth restriction.
448
449 We did not observe that circulating 25(OH) VD levels, the metabolite of VD
450 measured in clinical assessment, were changed with VD2 or VD3 treatment in
451 RUPP rats. We believe this could be due to the short duration of
452 supplementation, as clinical studies have shown that it takes several weeks for
453 lower doses of VD to be observed as increased circulating 25(OH) VD levels (2,
454 70). However, as there were no adverse effects on NP rats and there were
455 beneficial fetal and maternal improvements in RUPP rats, we believe that a lack
456 of increasing plasma VD levels demonstrates that VD supplementation is
457 promising even for patients that do not have a VD deficiency. Although VD2 and
458 VD3 are commercially available as supplements, clinical data have implicated
459 that VD3 may be a better therapeutic for humans (42, 61). In contrast, VD2 may
460 be more efficiently metabolized in rats (26). In accordance with these reports, our
461 data showed that VD2 lowered blood pressure, AT1-AA and sFlt-1 to a greater
462 extent than VD3 in RUPP rats. Importantly, the reductions of AT1-AA, sFlt-1 and
463 blood pressure were all in consistent proportion with regard to VD2 versus VD3,

464 further indicating that AT1-AA and sFlt-1 reduction led to the observed
465 attenuation of blood pressure in RUPP rats.
466
467 Our data demonstrate that VD reduces proinflammatory CD4+ T cell population,
468 inflammatory cytokines, AT1-AA, sFlt-1, ET-1, blood pressure and fetal demise in
469 RUPP rats, without adversely affecting maternal physiology or fetal development
470 in NP rats. Therefore, we conclude even in the absence of VD deficiency, VD
471 supplementation should be considered further as a safe preventative for
472 preeclampsia in pregnant women.
473
474 **Perspectives and Significance**
475 The present study demonstrates that VD could be a potential therapeutic to
476 improve pathological characteristics and hypertension associated with
477 preeclampsia without adverse fetal effects. Currently, clinical studies
478 investigating the potential of VD supplementation to improve PE have yielded
479 inconsistent results. However, as VD supplementation is a low-risk therapeutic, it
480 could provide an adjunct therapy for the pathogenesis associated with placental
481 ischemia. Further studies investigating VD supplementation in a large and
482 diverse population are needed. This study provides evidence that VD may
483 reduce pathological markers of PE, which will aid these future trials seeking to
484 comprehensively evaluate its therapeutic potential.
485

Acknowledgements

FUNDING SOURCES

This work was supported by NIH grants HL105324, HL124715, HL78147 and HL51971 and HD067541. RD is supported by the German Research Foundation (DFG, DE 631/9-1).

The authors report no conflict of interest

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Figure Legends

Figure 1

(a). VD2 and VD3 treatment in RUPP rats reduced blood pressure compared to RUPP rats. RUPP rats had higher blood pressure compared to NP. (*b).* Pup weights were not altered in NP rats with VD2 or VD3 treatment and were decreased in RUPP rat groups. *(c).* RUPP significantly increased fetal death compared to NP rats that was reduced with both VD2 and VD3 treatment, although only significantly with VD3. *(d).* Placental weights did not change with VD2 or VD3 treatment in either RUPP or NP. *(e).* Placental efficiency, evaluated as placenta/fetal weight ratio, did not change with Vitamin D treatment in either RUPP or NP groups. One-way ANOVA and Student's t-test, *P<0.05.

Figure 2

823 *(a).* CD4+ T cells were increased in RUPP rats compared to NP rats. VD2 and
824 VD3 decreased CD4+ T cells as percentage of total lymphocytes in RUPP rats,
825 although this was significant only with VD2. *(b).* FoxP3+ TREG cell percentage of
826 CD4+ T cells was decreased in RUPP rats compared to NP and was increased
827 modestly with VD treatment in both NP and RUPP rats. Student's t-test, *P<0.05.

828 **Figure 3.**

829 *(a).* RUPP rats had increased plasma TNF-α levels compared to NP rats and this
830 was decreased with both VD2 and VD3 treatment in RUPP rats, although these
831 changes did not reach significance due to high variation in the RUPP rat group.
832 *(b).* Plasma IL-6 levels were significantly higher in RUPP rats compared to NP
833 and significantly attenuated in both RUPP+VD2 and RUPP+VD3 groups. One-
834 way ANOVA and student's t-test.*P<0.05.

835 **Figure 4**

836 *(a).* Production of AT1-AA as assessed as beats per minute were greatly
837 decreased with VD2 and VD3 in RUPP rats. Furthermore, RUPP+VD2 rat AT1-
838 AA levels were significantly lower than RUPP+VD3 rats. One-way ANOVA and
839 student's t-test. *P<0.05. *(b).* B cells were increased in RUPP rats compared to
840 NP and were increased with VD2 and VD3 in NP rats but not significantly altered
841 in RUPP+VD2 or RUPP+VD3 rats. Student's t-test *P<0.05.

842 **Figure 5**

843 sFlt-1 was significantly increased in RUPP rats above levels of NP rats. sFlt-1
844 levels were significantly reduced in VD2-treated RUPP rats and modestly

reduced in VD3-treated RUPP rats. One-way ANOVA and student's t-test.*P<0.05.

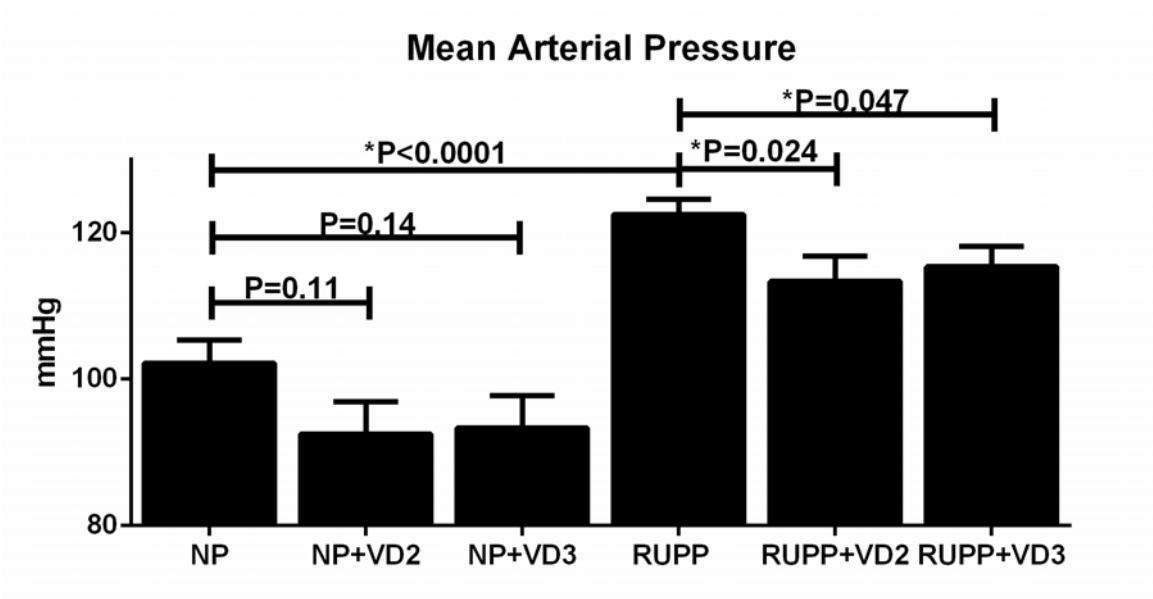
Figure 6

(a). Circulating nitric oxide levels as assessed by nitrate/nitrite concentrations in plasma were not altered with VD in RUPP rats. *(b).* Renal cortex expression if preproenndothelin-1, a precursor of endothelin-1, was significantly increased in RUPP rats compared to NP and attenuated with VD2 and VD3 treatment in RUPP rats. One-way ANOVA and student's t-test. *P<0.05.

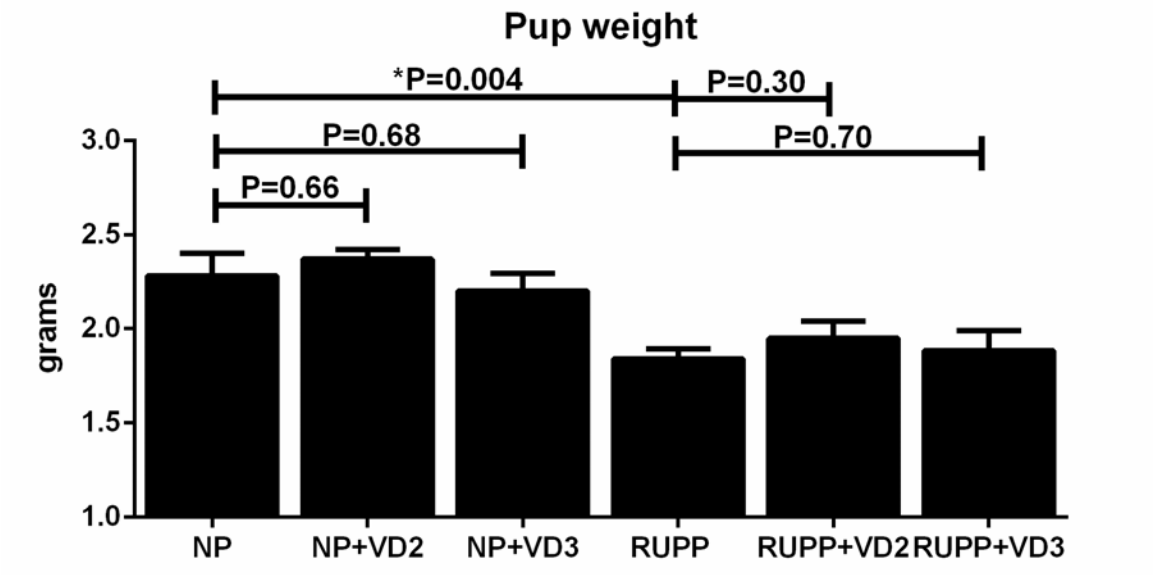
867

Figure 1

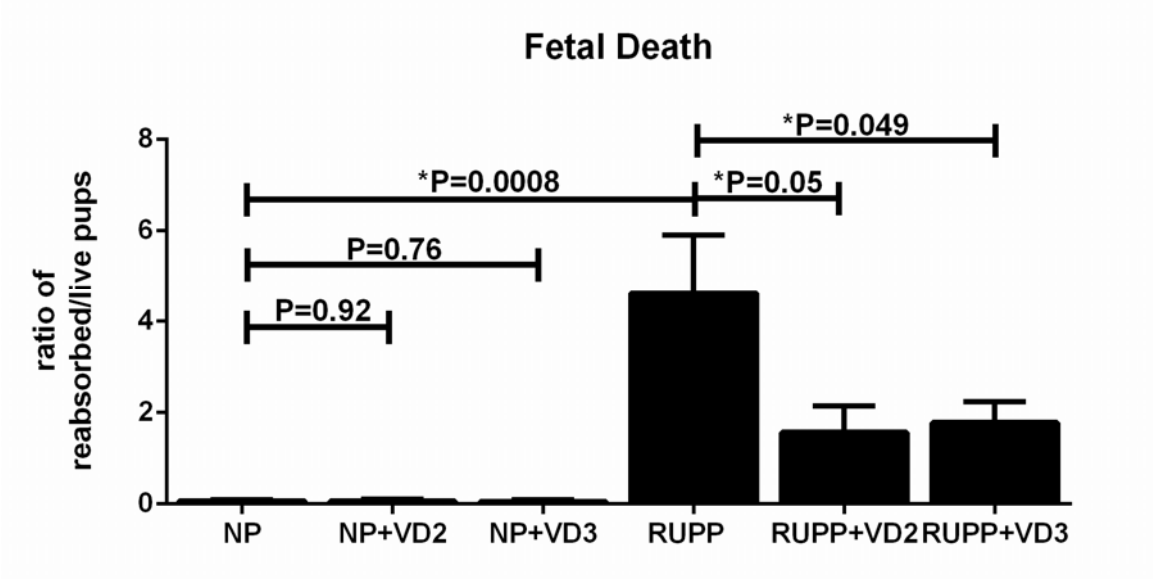
a.


Mean Arterial Pressure
*P=0.047
*P<0.0001
*P=0.024
P=0.14
P=0.11
mmHg
120
100
80
NP
NP+VD2
NP+VD3
RUPP
RUPP+VD2
RUPP+VD3

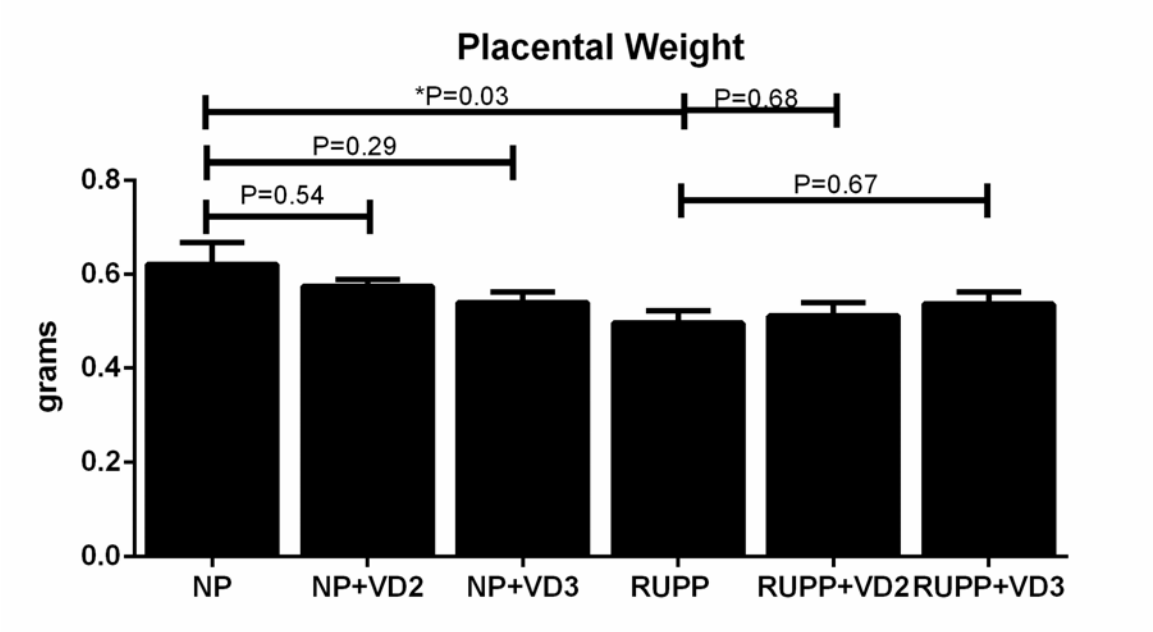
b.


Pup weight
*P=0.004
P=0.30
P=0.68
P=0.70
P=0.66
grams
3.0
2.5
2.0
1.5
1.0
NP
NP+VD2
NP+VD3
RUPP
RUPP+VD2
RUPP+VD3

c.


Fetal Death
ratio of reabsorbed/live pups
8
6
4
2
0
*P=0.049
*P=0.0008
*P=0.05
P=0.76
P=0.92
NP
NP+VD2
NP+VD3
RUPP
RUPP+VD2
RUPP+VD3

d.


Placental Weight
grams
0.8
0.6
0.4
0.2
0.0
*P=0.03
P=0.68
P=0.29
P=0.54
P=0.67
NP
NP+VD2
NP+VD3
RUPP
RUPP+VD2
RUPP+VD3

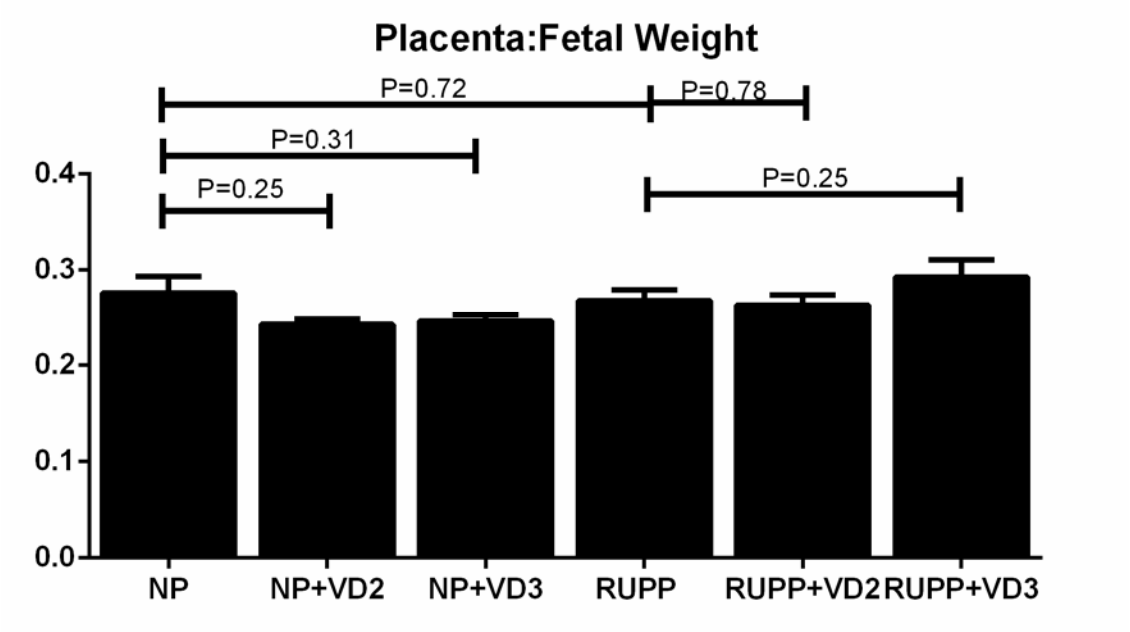
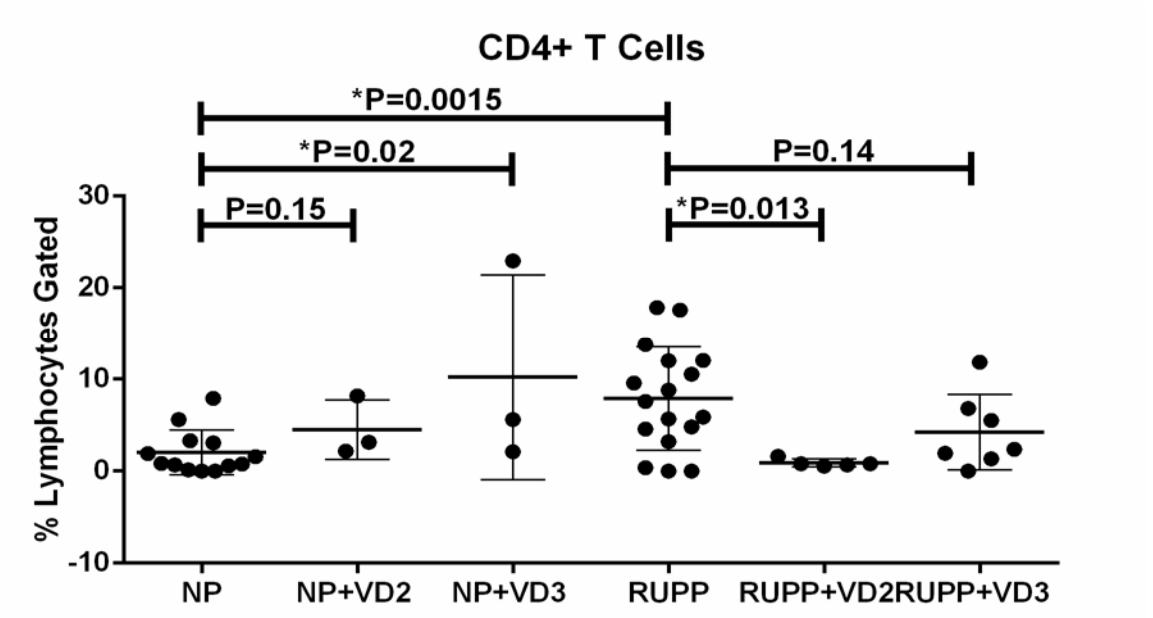

Placenta:Fetal Weight
P=0.72
P=0.78
P=0.31
P=0.25
P=0.25
0.4
0.3
0.2
0.1
0.0
NP
NP+VD2
NP+VD3
RUPP
RUPP+VD2
RUPP+VD3

Figure 2.

a.


CD4+ T Cells
*P=0.0015
*P=0.02
P=0.14
P=0.15
*P=0.013
% Lymphocytes Gated
30
20
10
0
-10
NP
NP+VD2
NP+VD3
RUPP
RUPP+VD2
RUPP+VD3

b.

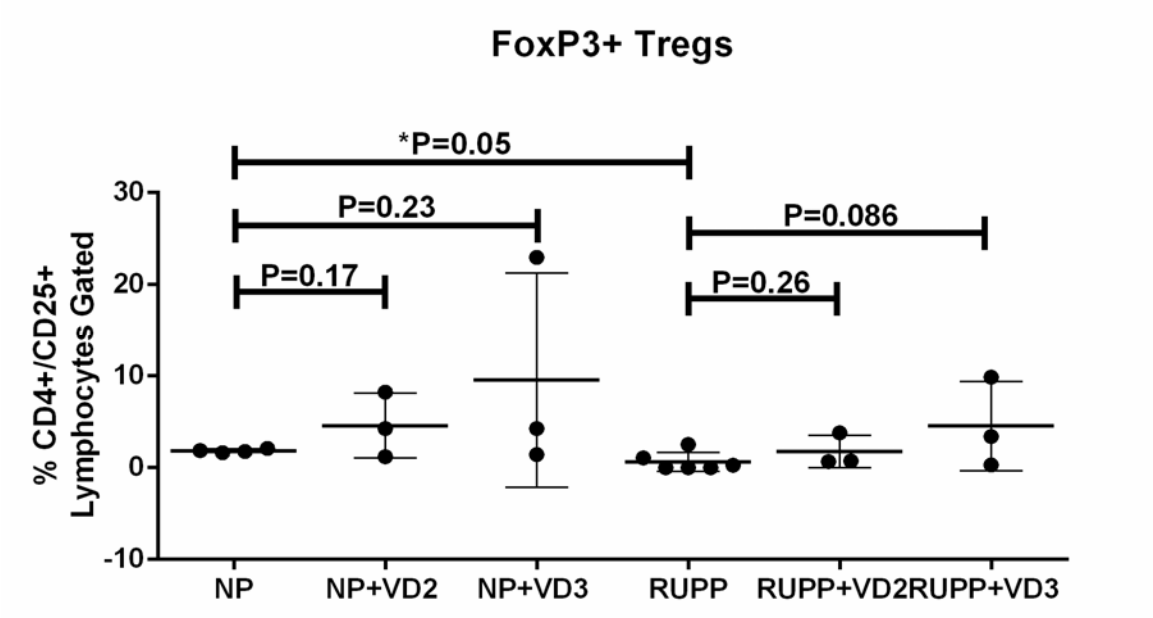
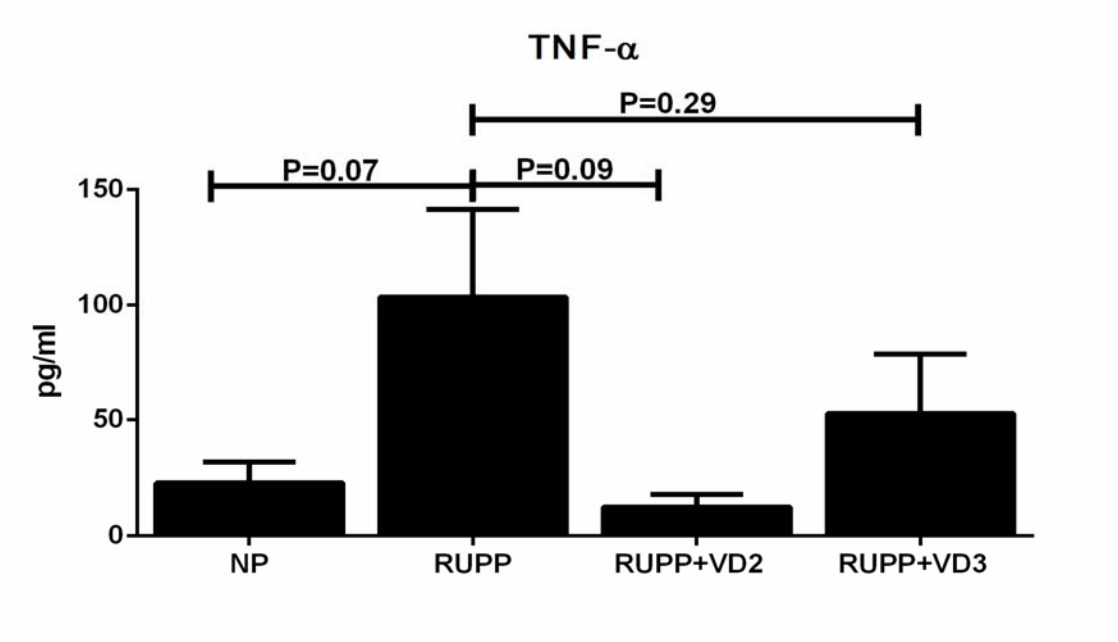

FoxP3+ Tregs
*P=0.05
P=0.23
P=0.086
P=0.17
P=0.26
% CD4+/CD25+ Lymphocytes Gated
30
20
10
0
-10
NP
NP+VD2
NP+VD3
RUPP
RUPP+VD2
RUPP+VD3

Figure 3.

a.


TNF-α
P=0.29
P=0.07
P=0.09
150
100
50
0
pg/ml
NP
RUPP
RUPP+VD2
RUPP+VD3

b.

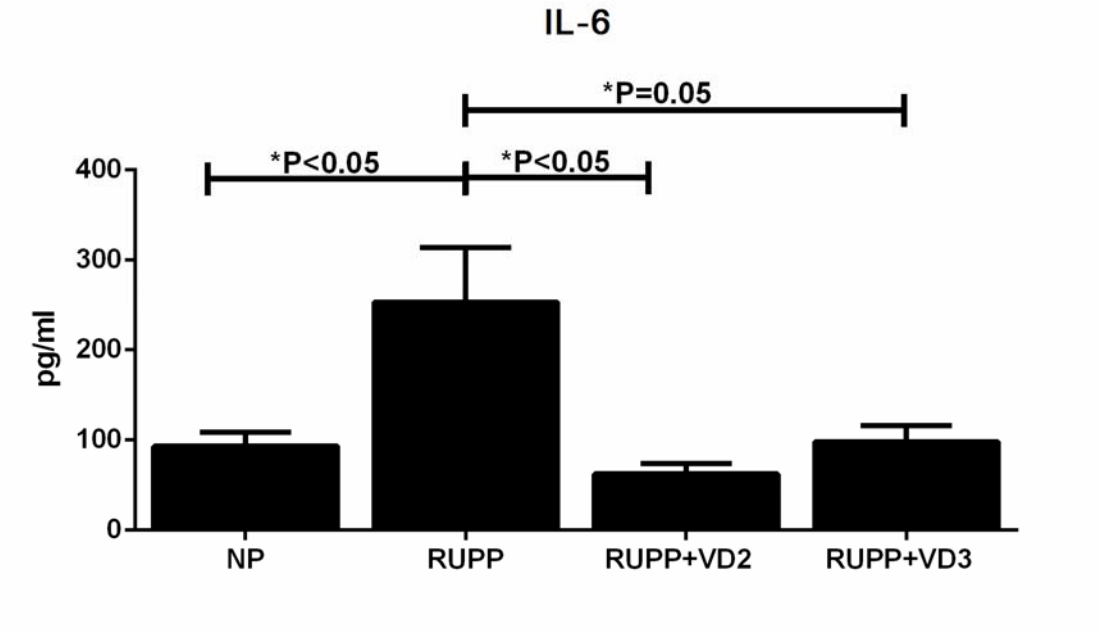
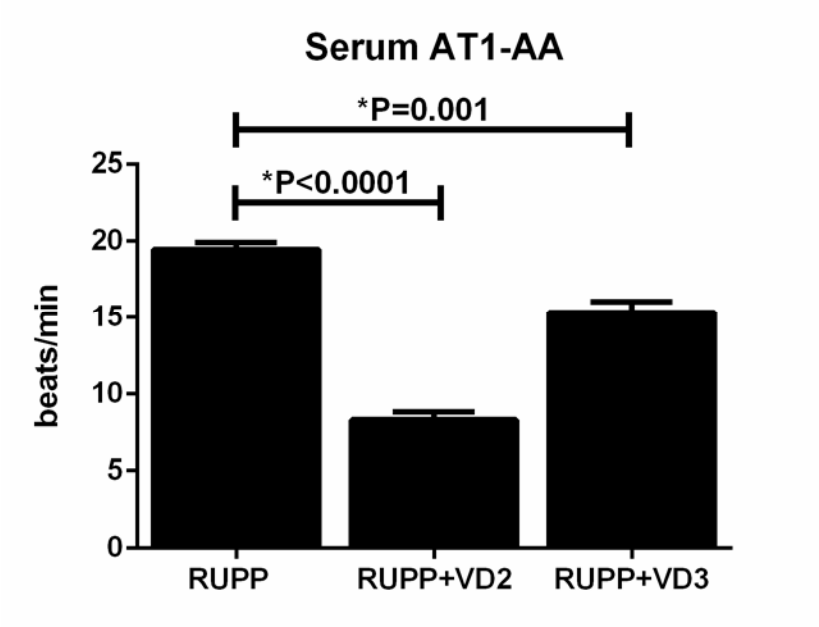

IL-6
*P=0.05
*P<0.05
*P<0.05
400
300
200
100
0
pg/ml
NP
RUPP
RUPP+VD2
RUPP+VD3

Figure 4.

a.


Serum AT1-AA
*P=0.001
*P<0.0001
beats/min
25
20
15
10
5
0
RUPP
RUPP+VD2
RUPP+VD3

b.

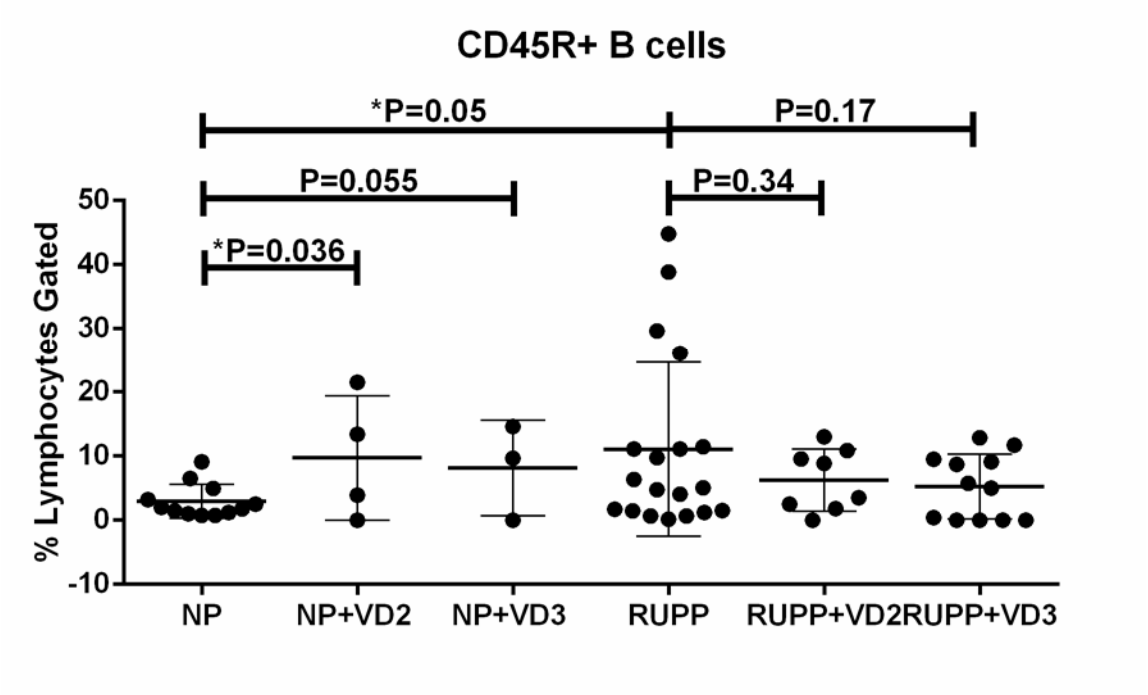

CD45R+ B cells
*P=0.05
P=0.17
P=0.055
P=0.34
*P=0.036
% Lymphocytes Gated
50
40
30
20
10
0
-10
NP
NP+VD2
NP+VD3
RUPP
RUPP+VD2
RUPP+VD3

Figure 5.

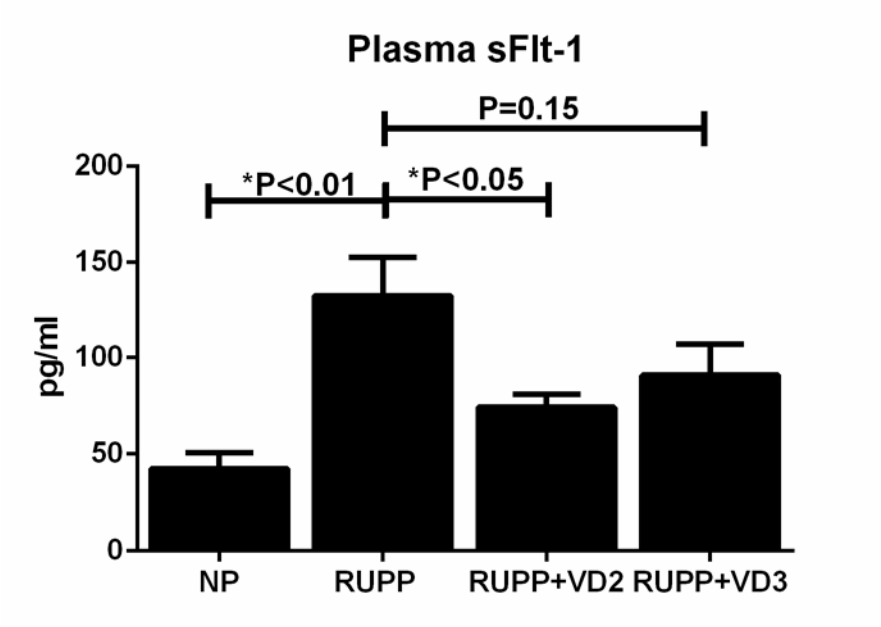
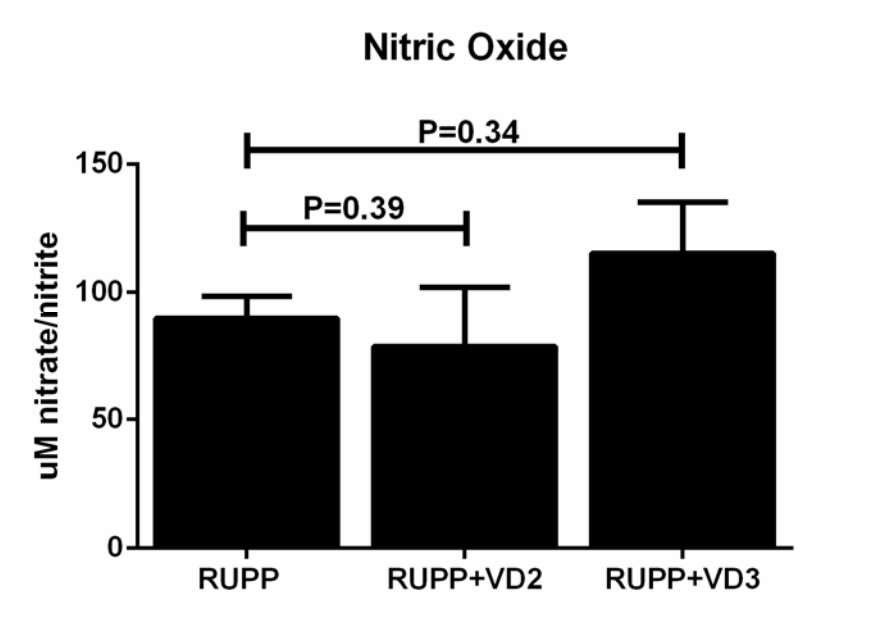
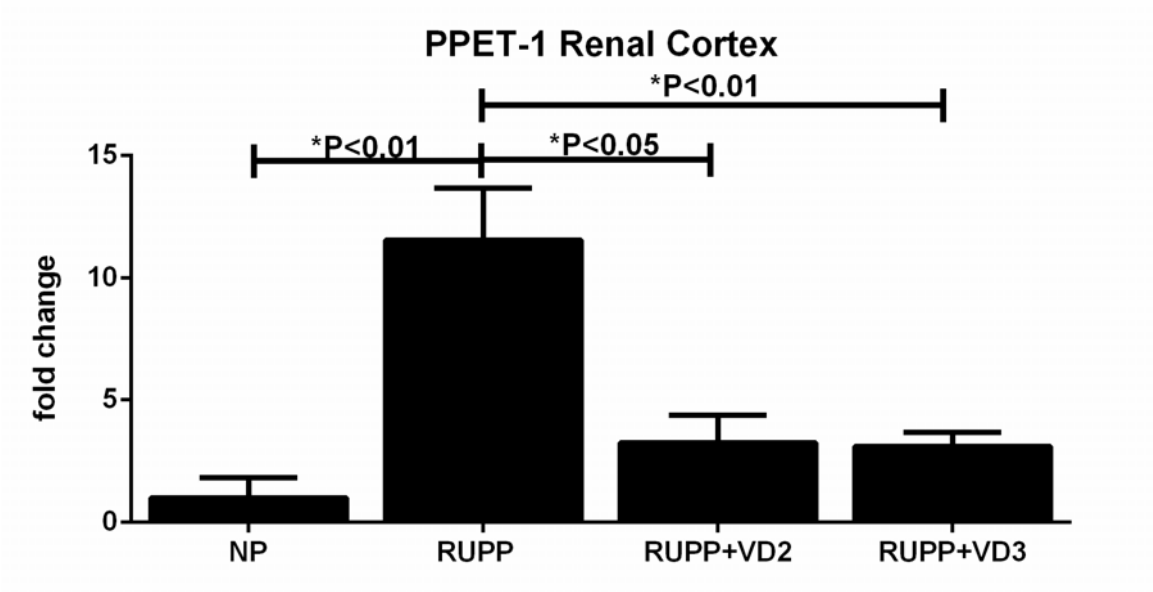

Plasma sFlt-1
P=0.15
*P<0.01
*P<0.05
200
150
100
50
0
pg/ml
NP
RUPP
RUPP+VD2
RUPP+VD3

Figure 6.

a.


Nitric Oxide
P=0.34
P=0.39
150
100
50
0
uM nitrate/nitrite
RUPP
RUPP+VD2
RUPP+VD3

b.


PPET-1 Renal Cortex
*P<0.01
*P<0.01
*P<0.05
15
10
5
0
fold change
NP
RUPP
RUPP+VD2
RUPP+VD3