Supplementary File

Hydrogen sulfide releasing molecule MZe786 inhibits soluble Flt-1 and prevents preeclampsia in a refined mouse RUPP model

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Materials and Methods:

Animal experiments:

Animal experimentation carried out in this study were approved and regulated by the UK Government Home Office in accordance with the 'Guidance on the operation of Animals' (scientific procedures) Act 1989 and in agreement with Aston University institutional guidelines and regulations for ethical animal use and care.

Acute blood flow assessment

The blood flow in the ovarian artery or abdominal aorta was assessed using laser Doppler flowmetry (LDF) (Kyocera Corp, JP) on mRUPP pre- and post-ligation as described previously (1). The changes in blood flow per unit time in the probed volume of the tissue were presented as blood flow rate (ml/min).

Blood Pressure Analysis and tissue collection

Blood pressure was measured on E17.5 as described previously(2). Following measurement, blood samples were collected, and the animals were euthanised. The individual yolk sac containing the embryos were separated from the uterus, the amniotic fluid was collected and the reabsorbed fetuses were counted. Their kidneys, and placentas were collected with part frozen in liquid N_2 for RNA analysis and the rest were fixed in 4% paraformaldehyde for histology.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay kits for murine sFlt-1, KIM-1 and VEGF were obtained from R&D Systems, UK and performed according to the manufacturer's specifications.

RNA extraction and real-time quantitative PCR

RNA was isolated from mouse placenta using RNeasy mini kit (Qiagen, UK) according to manufacturer's instructions. cDNA was subsequently generated from 1µg of starting mRNA using Transcriptor First Strand cDNA synthesis kit (Roche, UK) following manufacturer's protocol. Sample preparation and Real-time quantitative polymerase chain reaction was performed on a Roche LightCycler 480II (Roche, UK) using LightCycler DNA SYBR Green I according to manufacturer's guidance (Roche, UK).

List of primer sequences:

YWHAZ:

R 5' CCT CCA CGA TGA CCT ACG G 3'

F 5' TGA GCT GTC GAA TGA GGA GAG 3'

sFlt-1:

R 5' GGT ACA ATC ATT CCT CCT GC 3'

F 5' ATG CGT GCA GAG CCA GG AAC 3'

CSE:

R 5'-GGA AGT CCT GCT TAA ATG TGG TG-3'

F 5' TTC CTG CCT AGT TTC CAG CAT-3'

Immunohistochemistry

Murine kidney and placental tissue sections were prepared for immunohistochemistry as previously described (3). Biotin-labelled isolectin-B_4, anti-tyrosine (Abcam, UK) were used. The staining was imaged and analysed by NanoZoomer (Hamamatsu, Japan). For nitrotyrosine quantification, positive areas were marked and percent of positive area within total tissue area in the microscopic field was obtained for eight random fields along the kidney cortex using ImageJ software as described previously (4).

Tissue processing and histological analysis

Murine kidney and placental tissues were embedded with paraffin and stained for Hematoxylin and eosin (H&E). H&E stained kidney and placenta tissues were imaged using NanoZoomer (Hamamatsu, Japan). Area of the labyrinth zone was measured and analysed using ImageJ. Kidney analysis was done in a semi-quantitative manner as described previously (5, 6). Briefly, a virtual grid of fixed size of intersecting lines was overlaid on the images using Image J plug in (Grid_.class). Each sector of the grid was assessed for vascular congestion, fibrosis, tubular and glomerular degeneration. The number of sectors in each section that showed any abnormality was counted and was expressed as a percentage in relation to the total number of grid intersections covering the field. The severity grade was scored from 0-5 depending on the percentage of tissue affected. Specifically, the scores given were as in Table 1 (7). The H&E staining was done blindly by contract research laboratory Histologix, UK. Two researchers did analysis of placenta and kidney in blinded fashion.

Supplementary Figure Legends

Supplementary Figure 1. Comparison of conventional RUPP and refined RUPP model. A. Silver clips are placed around the aorta above the iliac bifurcation and the around the right and left ovarian arteries before the first segmental artery**. B.** In refined RUPP, the ovarian arteries on either side are ligated above the ovaries using 7-0 silk sutures **C.** Blood flow rate in the abdominal aorta at base line (pre-mRUPP) and after RUPP surgery (post-mRUPP). The blood flow rate in abdominal aorta remained unchanged post ligation. Data expressed as mean (±SEM), n=3.

Supplementary Figure 2. Semi-quantitative, blinded evaluation of kidney histology showing (**A)** a decrease in area of glomeruli and **(B)** increased arterial wall thickness in the kidney of mRUPP. The values did not reach significance. Data expressed as mean (±SEM) and analysed by Mann-Whitney U test, n=6. Clear circles represent sham and black squares represent mRUPP.

Supplementary Figure 3. (A) Mean arterial blood pressure (MAP) recorded at day 17.5 of gestation of Sham (n=15) or mRUPP mice. mRUPP mice were treated with control (n=15), 50mg/kg of MZe786 (MZe786 (D1)) (n=7) or 25mg/kg of MZe786 (MZe786(D2)) (n=6). 25mg/kg of MZe786 did not reduce elevated blood pressure in mRUPPs. (**B)** Live birth rate expressed as percentage in Sham and mRUPP treated with MZe786 or control. Data expressed as mean (±SEM) and analysed by 2-way ANOVA.

Supplementary Figure 4. Vascular endothelial growth factor (VEGF) levels in the maternal circulation. Surgically induced RUPP and Sham mice were treated with control (n=7) or MZe786 (n=7) and plasma levels of VEGF were determined using commercially available ELISA. Data expressed as mean (±SEM) and analysed by 2-way ANOVA.

Supplementary references

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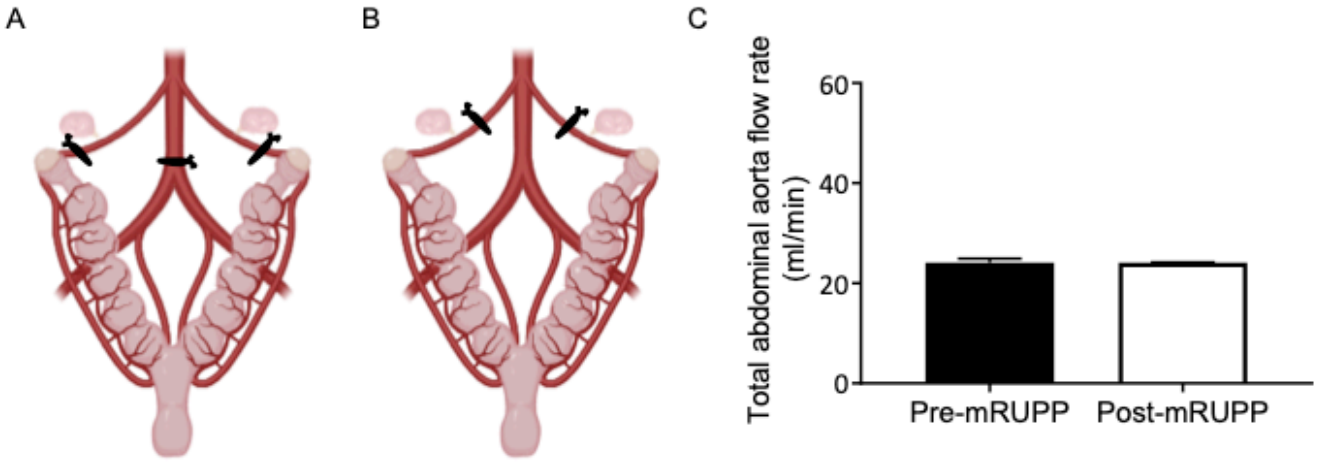
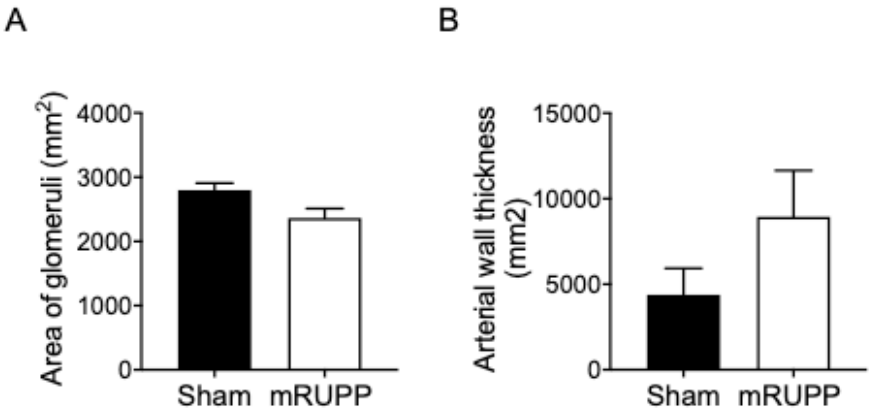
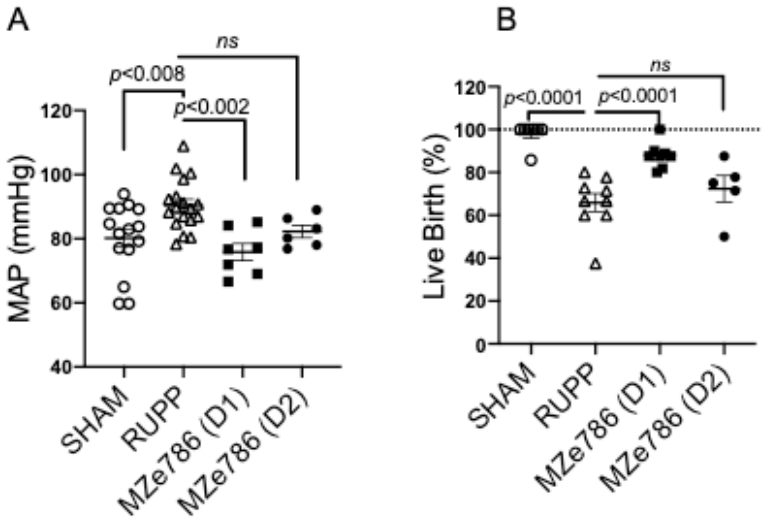
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4