Supplementary Material

Title: Tadalafil improves L-NG-nitroarginine methyl ester-induced preeclampsia with fetal growth restriction-like symptoms in pregnant mice.

Running head: Tadalafil improves preeclampsia in pregnant mice.

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Disclosure

The authors declared no conflict of interest.

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SUPPLEMENTARY INFORMATION

Supplementary Methods.

Measurement of systolic blood pressure (SBP).

SBP was measured at least three times for conscious mice using an indirect tail-cuff method (MK-2000; Muromachi Kikai Co., Tokyo, Japan)[1]. An average of three recordings were calculated for each measurement.

Evaluation of proteinuria.

Urine was obtained by gentle abdominal pressure 16 d.p.c. Proteinuria was semi-quantitatively assessed by assigning a score ranging from 0 to 3 based on results of a dipstick test (My Uri-Ace T®. Terumo, Tokyo, Japan)[2].

Placental growth factor (PlGF) and soluble fms-like tyrosine kinase-1 (sFlt-1) ELISA in the serum and the placenta.

Maternal serum samples from the dams 17 d.p.c. were collected and stored at –80°C before assays were conducted. One randomly chosen placenta was collected

from each dam. These placentas were homogenized by sonication in the presence of 50 mM Tris-HCl (pH 7.3), 100 mM NaCl, 5 mM EDTA, 1 mM EGTA (all from Sigma-Aldrich, St Louis, MO, US), and a protease inhibitor cocktail (Nacalai tesque, Kyoto, Japan). Homogenates were centrifuged at 10,000 × g and 4^{o}C for 10 min, and the supernatants were collected. Protein content was measured using the Qubit protein assay kit and Qubit 3.0 (Invitrogen, Carlsbad, CA, US), and samples were aliquoted and stored at –80^{o}C.

PlGF and sFlt-1 concentrations in the maternal serum and the placenta were determined using the Quantikine Elisa kit (R&D systems, Minneapolis, MN, US). Samples were assayed in duplicate.

Histopathological and immunohistochemical analysis of the placenta.

Placental tissue sections (3-μm thick) were stained using hematoxylin and eosin (HE), and the area of three placental layers (decidua, junctional zone, labyrinth zone) was measured using an image analyzer (DP 70 and DP controller; Olympus, Tokyo, Japan)[1]. Two or three placentas from each dam were randomly selected for histological

assessment.

Sections were incubated overnight at room temperature with rabbit polyclonal anti-αSMA antibody (1:200, Abcam, Cambridge, MA, US) to stain the endothelial cells of fetal capillaries in the labyrinth zone[3]. The sections were incubated with goat anti-rabbit IgG for 3 h before being incubated with peroxidase antiperoxidase complex for 2 h. Sections were incubated with 3,3'-diaminobenzidine (DAB) (DAB substrate kit; Vector Laboratories, Burlingame, CA, US). The centerlines of each of the maternal blood sinuses (αSMA-negative) and fetal capillaries (αSMA-positive) in the placental labyrinth zone were manually selected, and their widths were measured[4]. We analyzed 50 fetal capillaries and sinuses for 3–4 placentas per group.

Histopathological and immunohistochemical analysis of the kidney.

Kidney specimens were fixed in 4% paraformaldehyde (Nacalai tesque) in 0.01 M sodium phosphate buffer (PBS) (pH 7.4) and subsequently embedded in paraffin (Merck Ltd., Darmstadt, Germany) using standard procedures. Paraffin blocks were cut into 3-μm sections for periodic acid Schiff (PAS) staining to analyze sclerotic changes.

The sclerotic index was examined in 50 glomeruli randomly selected from each dam in a blinded manner. The sclerotic index was divided into five categories: 0 (no apparent damaged area), +1 (1–25% damaged area), +2 (26–50% damaged area), +3 (51–75% damaged area), and + 4 (76–100% damaged area)[5].

To evaluate oxidative stress in the kidney, after deparaffinization and rehydration, antigen was retrieved in 5% urea buffer by microwave heating for 5 min before being incubated in 1% H_2O_2 for 30 min to block endogenous peroxidase activity. Sections were incubated overnight at room temperature with mouse monoclonal anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) antibody (1:200; Japan Institute for the Control of Aging, Shizuoka, Japan). The sections were incubated in biotinylated anti-mouse IgG for 3 h before incubation with avidin-biotin complex (Vectastain ABC kit, Vector Laboratories) for 2 h. The sections were subsequently incubated with DAB (DAB substrate kit, Vector Laboratories). Glomerular 8-OHdG immunostaining was evaluated using a semiquantitative scoring system as described earlier[6].

Measurement of urinary cGMP and creatinine levels.

1 The cGMP concentrations were measured with a cyclic GMP radio immunoassay
2 kit (Yamasa, Tokyo, Japan) according to the manufacturer's instructions. Concentration
3 of urinary creatinine was determined by the Creatinine-Wako test (Wako).
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Supplementary Results

Table S1. Maternal body weight and food intake during pregnancy.

	C dam (n=6)	L dam (n=7)	TL dam (n=6)
Body weight at 11d.p.c. (g)	25.7 ± 1.0	26.3 ± 1.4	25.8 ± 1.0
Body weight at 13d.p.c. (g)	28.1 ± 1.5	26.3 ± 1.5	26.7 ± 1.0
Body weight at 17d.p.c. (g)	34.8 ± 1.6	31.6 ± 2.3*	32.3 ± 1.3
Maternal weight gain from 13 to 17 d.p.c. (g)	6.7 ± 0.8	5.3 ± 0.9*	5.6± 0.7
Food intake (g/day)	4.7 ± 0.6	4.0 ± 0.6	4.5 ± 0.6

* indicates $p<0.05$ compared to the control group by one-way ANOVA followed by Bonferroni's post-hoc test.

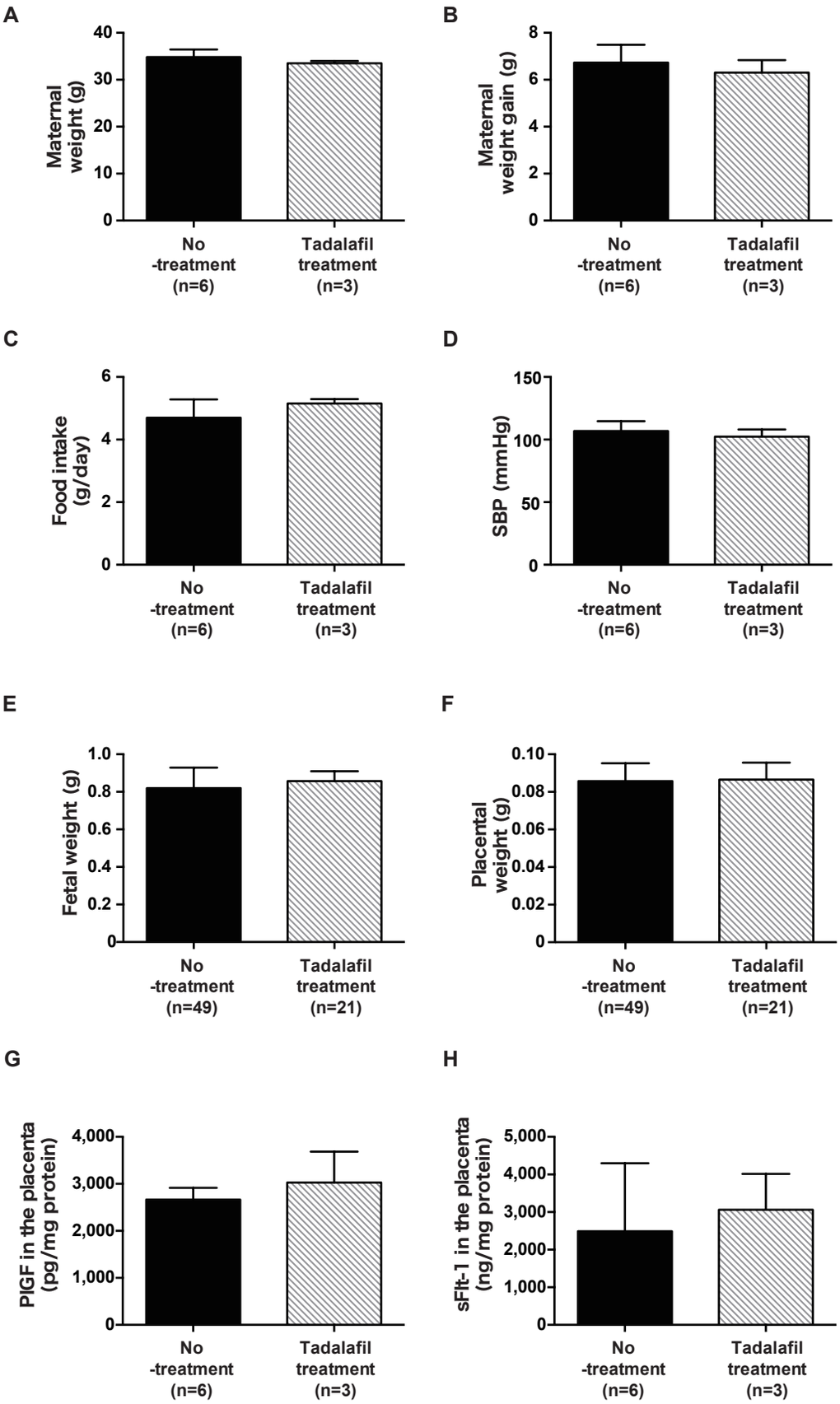

Figure S1

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Supplementary Figure Legends.

Table S1. Maternal body weight and food intake during pregnancy.

Figure S1. Maternal and fetal outcomes in normal pregnancy with tadalafil treatment.

The maternal body weight 17 d.p.c., (**A**), the maternal weight gain from 13 to 17 d.p.c. (**B**), the maternal daily food intake (**C**), the mean SBP 16 d.p.c (**D**), and PlGF and sFlt-1 concentrations of the placenta (**G** and **H**) of the dams in the no-treatment group (n=6) and the tadalafil-treated group (n=3). Fetal weight (**E**) and placental weight (**F**) of the no-treatment group (n=49) and the tadalafil-treated group (n=21). An unpaired Student's *t* test was used when comparing between the no-treatment group and the tadalafil-treated group.

Supplementary References

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