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RESEARCH ARTICLE

L-Citrulline ameliorates pathophysiology in a rat model of superimposed preeclampsia

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Background and Purpose: Preeclampsia, characterized by hypertension, proteinuria and restriction of fetal growth, is one of the leading causes of maternal and perinatal mortality. So far, there is no effective pharmacological therapy for preeclampsia. The present study was conducted to investigate the effects of supplementation with L-citrulline in Dahl salt-sensitive rats, a model of superimposed preeclampsia.

Experimental Approach: Parental Dahl salt-sensitive rats were treated with L-citrulline (2.5 $g \cdot L^{-1}$ in drinking water) from the day of mating to the end of lactation period. Blood pressure was monitored throughout pregnancy and markers of preeclampsia were assessed. Endothelial function of the pregnant Dahl salt-sensitive rats was assessed by wire myograph.

Key Results: In Dahl salt-sensitive rats, L-citrulline supplementation significantly reduced maternal blood pressure, proteinuria and levels of circulating soluble fms-like tyrosine kinase 1. L-Citrulline improved maternal endothelial function by augmenting the production of nitric oxide in the aorta and improving endothelium-derived hyperpolarizing factor-mediated vasorelaxation in resistance arteries. L-Citrulline supplementation improved placental insufficiency and fetal growth, which were associated with an enhancement of angiogenesis and reduction of fibrosis and senescence in the placentas. In addition, L-citrulline down-regulated genes involved in the TLR4 and NF-κB signalling pathways.

Conclusion and Implications: This study shows that L-citrulline supplementation reduced gestational hypertension and improved placentation and fetal growth in a rat model of superimposed preeclampsia. L-Citrulline supplementation may provide an effective and safe therapeutic strategy for preeclampsia that benefits both the mother and the fetus.

Abbreviations: ANGPT, angiopoietin; AUCC, area under the contraction curve; DBP, diastolic blood pressure; DSSR, Dahl salt sensitive rat; EDHF, endothelium-derived hyperpolarizing factor; EPR, electron paramagnetic resonance; FGF2, fibroblast growth factor 2; GD, gestational day; HIF-1α, hypoxia-inducible factor-1α; HT, hypoxanthine and thymidine; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; LKB1, liver kinase B1; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-monomethyl-L-arginine; MAP, mean arterial blood pressure; MMP9, metalloproteinase 9; mTOR, mammalian target of rapamycin; MYD88, myeloid differentiation factor 88; PLA2, phospholipase A2; PlGF, placental growth factor; PON2, paraoxonase 2; PRKAG2, protein kinase AMP-activated non-catalytic subunit gamma 2; SBP, systolic blood pressure; sFlt-1, soluble fms-like tyrosine kinase 1; THBS, thrombospondin; VASP, vasodilator-stimulated phosphoprotein; VCAM-1, vascular cell adhesion protein 1.

KEYWORDS
Dahl salt-sensitive rats, placental insufficiency, senescence, toll-like receptor, vascular function

1 | INTRODUCTION

Preeclampsia is a complication of pregnancy affecting 2% to 8% of all pregnancies worldwide and is a leading cause of maternal and perinatal mortality (Brown et al., 2018). Preeclampsia commonly occurs during the second half of pregnancy and is characterized by hypertension, proteinuria, damage to maternal organs and restriction of fetal growth (Foo et al., 2018). Preeclampsia can have long-term effects, increasing metabolic and cardiovascular risk to both mother and child. Women with a history of preeclampsia have approximately 2-fold increased risk of developing cardiovascular disease and around 10-fold increased risk of chronic kidney disease (Paauw et al., 2016). Fetal growth restriction limits the growth potential of the offspring and increases the risk of diseases later at adult age (Gluckman et al., 2008).

The origin of preeclampsia is unclear. The evidence, so far, shows the involvement of multifactorial mechanisms including an imbalance in angiogenic factors, aberrant inflammatory response, increased placental oxidative stress and placental ageing (Staff, 2019). The production of anti-angiogenic factors including the soluble fms-like tyrosine kinase 1 (sFlt-1) by the ischaemic placenta can cause endothelial dysfunction, intravascular inflammation and activation of the haemostatic system (Agarwal & Karumanchi, 2011). Due to the complex aetiology of preeclampsia and safety concerns on drug usage during pregnancy, there is still no effective pharmacological treatments available for preeclampsia (Brown et al., 2018). An ideal therapeutic agent should have protective effects in lowering blood pressure, ameliorating maternal phenotypes, and improving fetal growth and survival.

Preeclampsia can be considered as a vascular disorder. A functional and adequately vascularized placenta is crucial for a healthy pregnancy and birth outcome (Wu et al., 2004). Placental dysfunction results in a decrease in the angiogenic **vascular endothelial growth factor** (VEGF) and **placental growth factor** (PlGF) and the release of deleterious placental factors including sFlt1 into the maternal circulation causing generalized endothelial dysfunction (Gebara et al., 2021). **Nitric oxide** (NO) donors have potent vasodilator effects and have been shown to improve blood flow in the feto-placental circulation of pregnancies affected by mild preeclampsia (Luzi et al., 1999).

Several animal studies and clinical trials have tested the effect of **L-arginine**, a semi-essential amino acid and the substrate for vascular NO formation, in treating preeclampsia (Camarena Pulido et al., 2016; Oludare et al., 2018). Nevertheless, the bioavailability of arginine is relatively low, as 60% of oral arginine evades intestinal metabolism and another 9% is metabolized by liver (Weckman et al., 2019). **Citrulline** is the endogenous precursor to arginine and, compared with arginine, citrulline is more effective in augmenting NO levels, as it can bypass hepatic metabolism and is not metabolized by arginase (Weckman et al., 2019). Some studies suggested that the protective effect of citrulline treatment may not analogous with L-arginine treatment (Ham et al., 2015), and the two amino acids differentially regulate gene expression (Lee et al., 2018). Moreover, citrulline exerts anabolic effects on protein, which increases nitrogen balance in rats (Osowska et al., 2006) and enhances muscle protein synthesis in human (Jourdan et al., 2015), more efficiently than arginine. In addition, clinical use of citrulline showed an even greater safety profile than that of arginine, as none of the trials reported any adverse effects (Khalaf et al., 2019). Citrulline supplementation is reported to be safe and well tolerated as a single oral dose (2–15 g) in healthy adults (Schwedhelm et al., 2008). Indeed, citrulline has a limited degradation in the placenta and can be efficiently transferred from the mother to the fetus to facilitate fetal growth and development (Lassala et al., 2009). All these features make citrulline a good and safe candidate for treating preeclampsia. Nevertheless, the effects of maternal citrulline supplementation in targeting preeclampsia remain unclear.

Dahl salt-sensitive rats (DSSR) are a genetic model of salt-induced hypertension, renal injury, and insulin resistance (Rapp, 1982). Recent studies have reported the preeclamptic phenotype and fetal growth restriction in DSSR, which replicate many characteristics of

What is already known

- There is currently no effective pharmacotherapy available for preeclampsia.

What this study adds

- Citrulline supplementation attenuates placenta fibrosis and senescence and promotes placenta angiogenesis in an animal model of preeclampsia.
- Citrulline supplementation reduces maternal blood pressure and markers of preeclampsia, improves maternal endothelial function, and enhances fetal growth.

What is the clinical significance

- L-Citrulline may represent a potential therapy for human preeclampsia.

preeclampsia in humans (Dasinger et al., 2020; Gillis et al., 2015; Terstappen et al., 2019, 2020; Turbeville et al., 2019). During pregnancy, DSSR spontaneously display increased blood pressure, proteinuria and placental hypoxia and reduced uteroplacental blood flow. These phenotypes are associated with increased placental production of TNF-α, hypoxia-inducible factor-1α (HIF-1α), and sFlt-1 (Gillis et al., 2015). In the present study, we assessed the ability of citrulline to alleviate the pathophysiology of preeclampsia. We monitored blood pressure and measured the level of preeclampsia markers, placenta phenotypes, vascular function, and pregnancy outcomes in pregnant DSSR, with or without citrulline supplementation.

2 | METHODS

2.1 | Animals

All animal care and experimental procedures were in accordance with the German animal protection law and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and were approved by the responsible regulatory authority (Landesuntersuchungsamt Rheinland-Pfalz; 23 177-07/G 16-1-038). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020).

The rats (DSSR; RRID:RGD_2308886) were from Charles River Laboratories (Sulzfeld, Germany) and were kept in adequate groups provided with nesting material and enrichment. F0 parental DSSR were fed with standard chow diet ad libitum. Only the females giving the first birth were used in the study. Adult female rats from the same litter were assigned to individual cages. The rats were randomized to receive either normal water or L-citrulline (2.5 $g \cdot L^{-1}$ in drinking water) from the day of mating (at the age of 12 weeks) to the end of lactation period. Pregnancy was confirmed by checking the plug and timed from that day, in gestational days (GD). Citrulline was replaced every 2 days during the treatment period. Rats from both groups were studied either during pregnancy (9 pregnant rats per group at GD12; 10 pregnant rats per group at GD21) or postpartum (4 litters with 24 pups in total in the control group and 5 litters with 36 pups in total in the citrulline group). Equal numbers of female rats were set for mating to generate groups of equal size. The differences in numbers of individual used in this study were due to some unsuccessful matings. On the last day of the experiments, isoflurane and injection of pentobarbital i.p. were used to kill the animals. In the GD12 experiments, the litter size and embryo weight was measured and the aorta and mesenteric arteries collected to assay vascular function ex vivo. In the GD21 experiments, blood pressure of the pregnant rats was measured during each week of pregnancy. On GD21 the rats were killed, the litter size and embryo weights were measured, urine and serum samples were collected, and umbilical vein taken for ex vivo vascular function experiments and placenta samples for Western blotting, qPCR, and staining. For postpartum experiments, the body weights of the pups were measured till day 8. The sizes of groups were calculated so that the minimal necessary numbers of animals are used.

2.2 | Blood pressure measurement

Mean blood pressure were measured non-invasively in conscious rats (control = 6, citrulline = 7) by a computerized system (CODA Monitor, Kent Scientific, Torrington, CT, USA) as described in our previous studies (Man, Chen, Wu, et al., 2020; Man, Chen, Zhou, et al., 2020; Wu et al., 2014). Rats were restrained in individual holders. The occlusion cuff and the volume-pressure recording cuff were placed close to the base of the tail. After an adaptation period of 30 min on a warm pad (37°C), 10 preliminary measurements were performed before actual measurement. Rats were acclimated for three consecutive days prior to the actual measurement. Results are presented as the mean of at least 15 recordings on each occasion taken. The measurements were performed at the same time of a day from 2 PM to 4 PM by the same investigator.

2.3 | Isometric tension studies

The vascular function of the second-order mesenteric arteries and umbilical veins was accessed by wire myograph as described in our previous studies (Man, Chen, Zhou, et al., 2020). Briefly, vessels were dissected free of adherent connective tissues and placed in cold modified Krebs–Ringer bicarbonate buffer (composition in mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 11.1 D-glucose; pH 7.4) gassed with 95% O_2/5% CO_2. Vessel rings (2–3 mm long) were suspended in the chambers of a Mulvany–Halpern wire myograph system (620 M, Danish Myo Technology A/S, Aarhus, Denmark). Isometric force was recorded by a PowerLab/8SP system (AD Instruments Inc., Colorado Springs, CO, USA). The preparations were equilibrated for 30 min at the optimal resting tension. For mesenteric arteries, the viability of the endothelium was tested by the relaxation response to a single dose of acetylcholine (10^{-4}M), after obtaining a reference contraction to 60-mM KCl twice, prior to the actual experiment. For actual experiment, preparations were incubated for 30 min with or without different inhibitors (10^{-4} M L-NAME, and 10^{-5} M indomethacin). Preparations were then pre-contracted by exposing to increasing concentrations of phenylephrine (10^{-9} to 10^{-5} M). Endothelium-dependent relaxation was examined by exposure to increasing concentrations of acetylcholine (10^{-9} to 10^{-4} M). Change in tension is expressed as percentage of the contraction to phenylephrine (~80% of the reference KCl contraction). For umbilical veins, preparations were incubated for 30 min with or without 10^{-4} M L-NAME. Contraction was examined by exposure to increasing concentrations of acetylcholine (10^{-9} to 10^{-4} M). Area under the contraction curve (AUCC) was measured in different concentration-dependent curves of the preparation, with or without L-NAME. The difference (ΔAUCC) between AUCC of the curve with L-NAME and without L-

NAME was calculated. The effect of L-NAME was presented as the ΔAUCC with reference to the control group for comparison.

2.4 | Serum supplementation in cell culture for senescence assay

Human umbilical vein endothelial cells (HUVEC)-derived EA.hy 926 cells (RRID:CVCL_3901) were cultured in basal medium consisting of DMEM (Sigma-Aldrich, Taufkirchen, Germany) with 10% fetal calf serum (FCS; PAA Laboratories, Cölbe, Germany), 2-mM L-glutamine, 2-mM sodium pyruvate, 1% penicillin/streptomycin, and 1% hypoxanthine and thymidine (Life Technologies/Thermo Fisher Scientific, Waltham, MA, USA). For the senescence assay, EA.hy 926 cells were incubated in basal medium alone or with basal medium supplemented with 4% serum from either control or citrulline-treated rats. After culture for 3 days, cells were either collected for RNA extraction or were fixed and stained with senescence detection kit (ab65351, Abcam, Cambridge, UK). This kit uses β-galactosidase (SA-beta-Gal) activity which is known to be restricted to senescent cells. In brief, the cells were fixed in fixative solution for 15 min and stained with SA-beta-Gal solution at 37°C overnight. The development of the bluish-green colour was observed using a light microscope. These experiments were repeated three times, independently.

2.5 | Gene expression studies by quantitative PCR

Total RNA from randomly selected placentas from different rats and cell culture was isolated using peqGOLD TriFast™ (PEQLAB, Erlangen, Germany), and cDNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA), as described earlier (Xia et al., 2017). QPCR was performed using SYBR Green JumpStart™ *Taq* Ready-Mix™ (Sigma-Aldrich) on an iCycler Real-Time PCR Detection System (Bio-Rad, Waltham, MA, USA). Quantification was achieved by the difference in quantification cycles (ΔΔCt) values that were normalized to reference genes (GAPDH for rat samples or β-actin for EA-hy926 samples). Relative gene expression of target gene in each sample was expressed as the percentage of control. Specificity of the qPCR primers were checked by melting curve analysis or gel electrophoresis of the qPCR product. The sequence of the primers used are listed in Table S1.

2.6 | Protein expression by Western blotting

A subset of randomly selected aorta and placenta samples were homogenized in RIPA buffer with 1% (v/v) proteinase inhibitor cocktail (#78442, Thermo Fisher Scientific). Equal amounts of lysate protein was loaded and separated in SDS-PAGE. The resolved proteins were transferred onto nitrocellulose membranes and probed with specific primary antibody at 4°C overnight with agitation. GAPDH or β-actin was probed as a loading control. The protein bands were visualized using enhanced chemiluminescence (ECL) reagents (GE Healthcare, Chicago, IL, USA) and developed in Fujitsu Biomedical film (Fujitsu, Tokyo, Japan). Quantification protein expression was based on the ratio of target protein to GAPDH. The following primary antibodies were used: anti-CD31 (Invitrogen, Waltham, MA, USA; #PA5-16301, 1:1000, RRID: AB_10981955), anti-PlGF (#PA5-79814, Invitrogen, 1:1000, RRID: AB_2746929), anti-VEGF (#MA1-16629, Invitrogen, 1:1000, RRID: AB_2212682), anti-p-eNOS (Cell Signaling, Danvers, MA, USA; #9571S, 1:1000, RRID:AB_329837), anti-eNOS (#610297, BD, 1:1000, RRID: AB_397691), anti-p-VASP (New England Biolabs, Ipswich, MA, USA; #3114S, 1:1000, RRID:AB_2213396), anti-VASP (#3132S, New England Biolabs, 1:1000, RRID:AB_2213393), anti-β-tubulin I (#T7816, Sigma-Aldrich, 1:30,000, RRID:AB_261770), and anti-GAPDH (Epitomics, Burlingame, CA, USA; #2251-1, 1:30,000, RRID:AB_1267174). The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.7 | Masson's trichome staining

Placentas were fixed in 4% paraformaldehyde and embedded in paraffin. Microtome sectioning was performed to obtain slides with thickness of 5 μm. After deparaffination and rehydration, Trichrome Stain Kit (Abcam) was used to analyse collagen fibres according to manufacturer's instructions. In brief, the slides were incubated in the preheated Bouin's Solution at 60°C for 1 h. The yellow colour on the slides was removed by rinsing in running tap water followed by staining in Working Weigert's Iron Haematoxylin Solution. The slides were then rinsed in deionized water and stained in Working Phosphomolybdic/Phosphotungstic Acid Solution followed by Aniline Blue Solution and 1% acetic acid. The slides were then rinsed, dehydrated, and mounted. Collagen staining of images was quantified by NIH ImageJ software. Staining was performed in six different placenta samples from each group.

2.8 | Immunohistochemical (IHC) staining

Placentas were fixed in 4% paraformaldehyde and embedded in paraffin. Microtome sectioning was performed to obtain slides with thickness of 5 μm. After deparaffination and rehydration, slides were immersed in warm EnVision FLEX target Retrieval Solution (#K8004, Agilent, Santa Clara, CA, USA) for antigen retrieval. The slides were then immersed in peroxidase blocking solution to inhibit the activity of peroxidase. The sections were incubated with primary antibody to CD31 (#PA5-16301, Invitrogen, 1:50, RRID:AB_10981955) in 4°C overnight. After washing twice in phosphate buffered saline with 0.1% Tween 20 (PBST), the sections were incubated with HRP-linked anti-rabbit antibody (#K4002, Agilent, RRID:AB_2630375) at room temperature for 1 h. After washing twice in PBST, the sections were incubated with DAB (3,3′-diaminobenzidine) staining kit (#K3468, Agilent, USA) for 5 min at room temperature. The slides were rinsed in distilled water, followed by dehydration and mounting.

2.9 | ELISA assay

Serum level of rat soluble fms-like tyrosine kinase-1 (sFlt-1, #MBS2602003) and placenta growth factor (PIGF, #MBS026910) were examined using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction (Mybiosource, San Diego, CA, USA). In brief, 100-μl serum was added as samples. Samples and standard were then incubated at room temperature for 90 min with gentle shaking. After washing twice, biotinylated antibodies were added and incubated at room temperature for another 60 min. After washing for three times, HRP-avidin was added and incubated for 30 min at room temperature with gentle shaking. After washing for five times, colour reagent was added in dark with gentle shaking. After 30 min of incubation, stop solution was added and the absorption was read at 450 nm immediately with Sunrise™ microplate reader (Tecan Group, Männedorf, Switzerland) and analysed by Magellan™ software (Tecan Group, Switzerland).

2.10 | Urinary biochemical assays

Urine samples were collected directly from the bladder, immediately after killing. Urine creatinine was measured by a blood chemical analyser (Reflotron, Roche Co., Basel, Switzerland) using the specified analysis kits supplied from the manufacturer. Urine protein was measured by bicinchoninic acid protein assay.

2.11 | In vitro NO production assays

Electron paramagnetic resonance (EPR)-based NO-trapping technique with iron-diethyldithiocarbamate (Fe $(DETC)_2$) colloid was used to assess the NO production in rat aortas in vitro, as previously described (Munzel et al., 2017). The aorta was cut into rings of 3 mm in length. The samples were incubated with 10-μM calcium ionophore (A23187) and 200-μM Fe $(DETC)_2$ colloid solution at 37°C for 60 min in Krebs–HEPES buffer. EPR conditions: $B_0 = 3276$ G, sweep = 115 G, sweep time = 60 s, modulation = 7000 mG, MW power = 10 mW, gain = 9×10^2 using a Miniscope MS400 from Magnettech (Berlin, Germany).

2.12 | HPLC for detection of arginine and citrulline concentrations

Arginine and citrulline concentrations were measured in rat sera by high-performance liquid chromatography (HPLC) using precolumn derivatization and fluorescence detection. For protein precipitation, 225-μl ice-cold ethanol (final concentration 70%) was added to 50-μl rat serum and centrifuged at 4°C, 14000 *g* for 15 min. Supernatants were then supplemented with 50-μl 2-N HCl, 750-μl 1× PBS (pH 2), and 4 nmol N^G-monomethyl-L-arginine (L-NMMA) as external standard, before application to a Strata™-X-C 33-μm polymeric strong cation column (Phenomenex, Aschaffenburg, Germany, #8B-S029-TAK). Citrulline recovery (85–87%) was enabled by low sample pH and validated by adding standards. For removing other amino-acids and unwanted substances, the column was washed with 1-ml 0.1-N HCl and methanol, respectively. Basic amino acids, such as arginine, L-NMMA and citrulline, were eluted with 1-ml methanol: water:25% NH_3 (5:4:1), vacuum-dried, and resuspended in 200-μl 0.5-M borate buffer pH 9.5. Samples of eluate (50 μl) were incubated with 12-μl OPA reagent (20-mg *o*-phthaldialdehyde, 1.8-ml methanol, 200-μl 0.5-M borate buffer pH 9.5, and 20-μl β-mercaptoethanol) for 120 s before 22-μl 1-N acetic acid were added. A volume of 10 μl was then separated on a XSelect CSH column (C18, 4 μm 4.6 × 150 mm, Waters, Eschborn, Germany) using a gradient of 50-mM sodium acetate, pH 6.8 (0′:87%, 10′:86%, 29′:84%, 30′:70%, 33′:0%, 37′:0%, 42′:87%) and acetonitrile (complementary to 100%, respectively, flow rate 0.8 ml/min, 35°C). Fluorescence (excitation wavelength 330 nm, emission wavelength 450 nm) was monitored with a Shimadzu RF-20A fluorescence detector and quantified using the analysis program McDAcq (Bischoff Chromatography, Leonberg, Germany).

2.13 | Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Statistical analysis was undertaken only for studies where each group size was at least $n = 5$ and the data analyses were blinded. The group size is the number of independent values, and statistical analysis was done using these independent values. Results are expressed as mean ± SEM, with individual values shown or with the *n* values stated in the figure legends. mRNA expression data were normalized to control or baseline to show the relative changes. Student's *t* test was used for comparison between two groups and one-way ANOVA was used for comparison between multiple groups. *P* values < 0.05 were considered significant. GraphPad Prism 9.0.1 (GraphPad Software, La Jolla, CA, USA) was used to generate graphs and statistical analysis.

2.14 | Materials

L-Citrulline, phenylephrine, acetylcholine, indomethacin, L-NAME and L-NMMA were supplied by Sigma-Aldrich. (Fe $(DETC)_2$) colloid was generated by mixing equal volumes of sodium diethyldithiocarbamate (Sigma-Aldrich) solution (0.45 mg ml^{-1}) and $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich) solution (0.28 mg ml^{-1}).

2.15 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and

are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Cidlowski, et al., 2021; Alexander, Fabbro, et al., 2021a, b; Alexander, Mathie, et al., 2021).

3 | RESULTS

3.1 | Citrulline reduces preeclamptic phenotypes during pregnancy in DSSR

We investigated the effect of maternal L-citrulline supplementation in the pregnant DSSR. Arterial blood pressure was monitored from the day of mating to the third week of pregnancy. In the control DSSR, the blood pressure increased after pregnancy, with significantly higher systolic blood pressure (SBP) at week 1, significantly higher diastolic blood pressure (DBP) at weeks 1, 2, and 3, and significantly higher mean arterial blood pressure (MAP) at weeks 2 and 3, than before mating (Figure 1a–c). In contrast, a trend of blood pressure reduction was observed in the pregnant DSSR treated with citrulline, with a statistically significant decrease of SBP at week 3 (Figure 1a). When comparison is performed between the two groups, both SBP and DBP were lower in the citrulline-treated DSSR than the control DSSR at weeks 1, 2, and 3 after pregnancy (Figure 1a–c). The statistical significance of the difference between the two groups became even more pronounced, when the changes of blood pressure were compared (Figure 1d–f).

It is known that the DSSR exhibit an increase in proteinuria over the course of pregnancy (Gillis et al., 2015). Urinary protein-to-creatinine ratio was significantly reduced by citrulline supplementation (Figure 1g), as was the circulating level of sFlt-1 (Figure 1h),

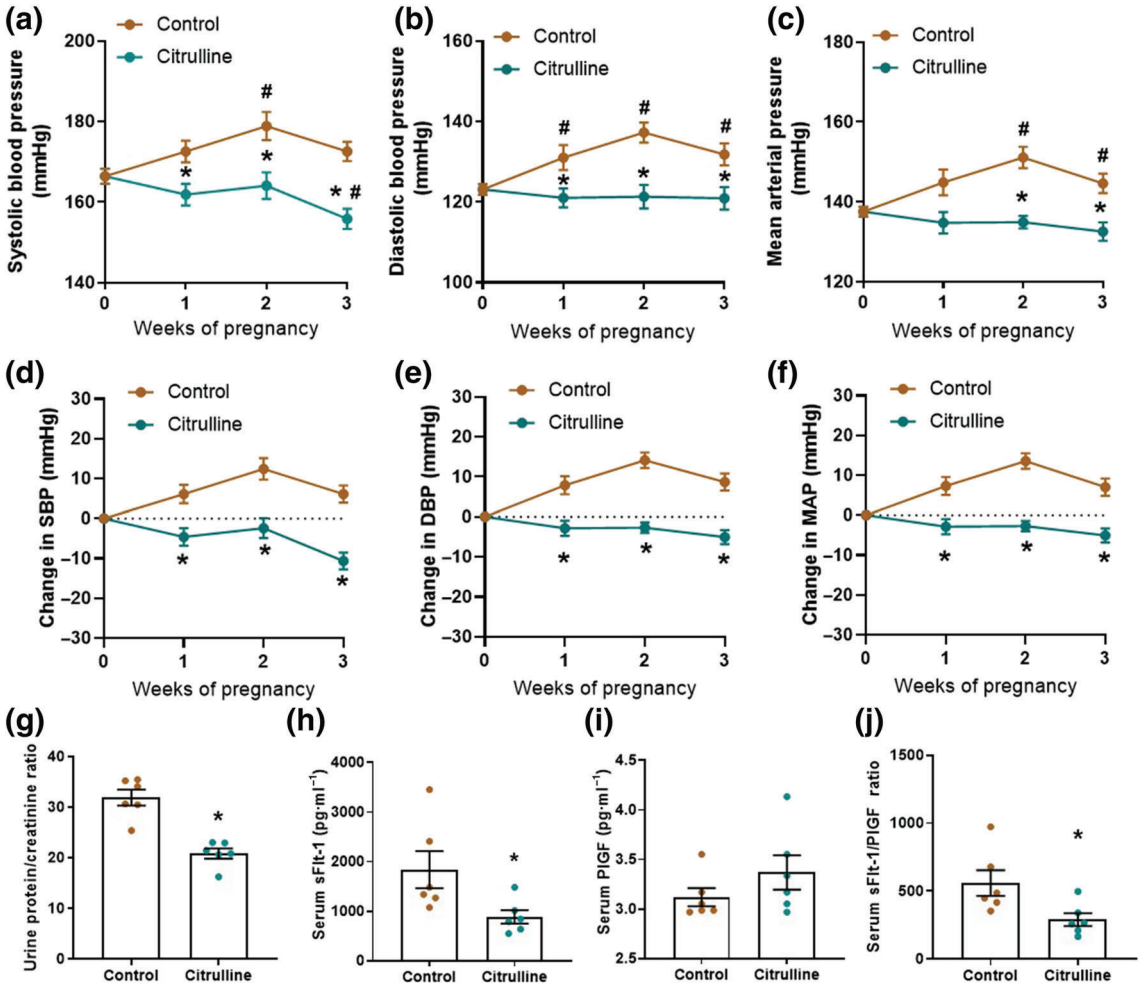

FIGURE 1 Citrulline reduces preeclamptic phenotypes in DSSR. L-Citrulline (2.5 g·L^{-1} water) was administered from the day of mating to the end of the experiments. Arterial blood pressure was measured by tail-cuff method in pregnant DSSR (a–c). Changes in systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial blood pressure (MAP) during pregnancy compared with baseline (week 0) were calculated (d-f). $N = 6$ (Control) or 7 (Citrulline). The experiment was terminated at gestational day (GD) 21. The urinary protein content was measured by bicinchoninic acid assay, and creatinine level was measured by a chemical analyser. The urine protein-to-creatinine ratio was calculated (g). Levels of soluble fms-like tyrosine kinase-1 (sFlt-1) (h) and placental growth factor (PlGF) (i) were measured in serum collected at GD21 with specific ELISAs. The serum sFlt-1-to-PlGF ratio was calculated as a preeclampsia marker (j). Data are presented as means ± SEM (a-f) or with individual values (g-j). *P < 0.05, significantly different from control. $^{\#}P$ < 0.05, significantly different from baseline value of the same group

whereas serum level of PlGF was not significantly different between control and citrulline-treated DSSR (Figure 1i). Citrulline supplementation reduced the serum sFlt-1/PlGF ratio at late pregnancy of DSSR (Figure 1j).

3.2 | Citrulline improves placental insufficiency during pregnancy and improves fetal growth in DSSR

Placental insufficiency causes intrauterine growth restriction during preeclampsia, which leads to the reduction of fetus size in both humans and in animal models (Pierik et al., 2019). Citrulline supplementation significantly improved fetal growth in DSSR. At GD12, pregnant DSSR were killed to assess intrauterine growth. The number of embryos per litter was not significantly different in the control and citrulline-treated DSSR (Figure 2a), but citrulline-treated DSSR had significantly larger embryos, as measured by increased weight (Figure 2b) and size (Figure 2c). At GD21, the number of fetuses per litter was not significantly different between control and citrulline-treated DSSR (Figure 2d). Placental efficiency, as quantified by the ratio of pup-to-placenta weight, was improved by citrulline supplementation (Figure 2e). A high placenta weight-to-pup weight ratio is usually associated with growth restriction and represents a reduced nutrient transport capacity of the placentas.

The umbilical vessels carry the blood supply of the fetus, and preeclampsia may lead to reduced placental perfusion and impaired fetal development. Responses to acetylcholine were studied in the umbilical vein, using in the wire myograph system. Acetylcholine induced

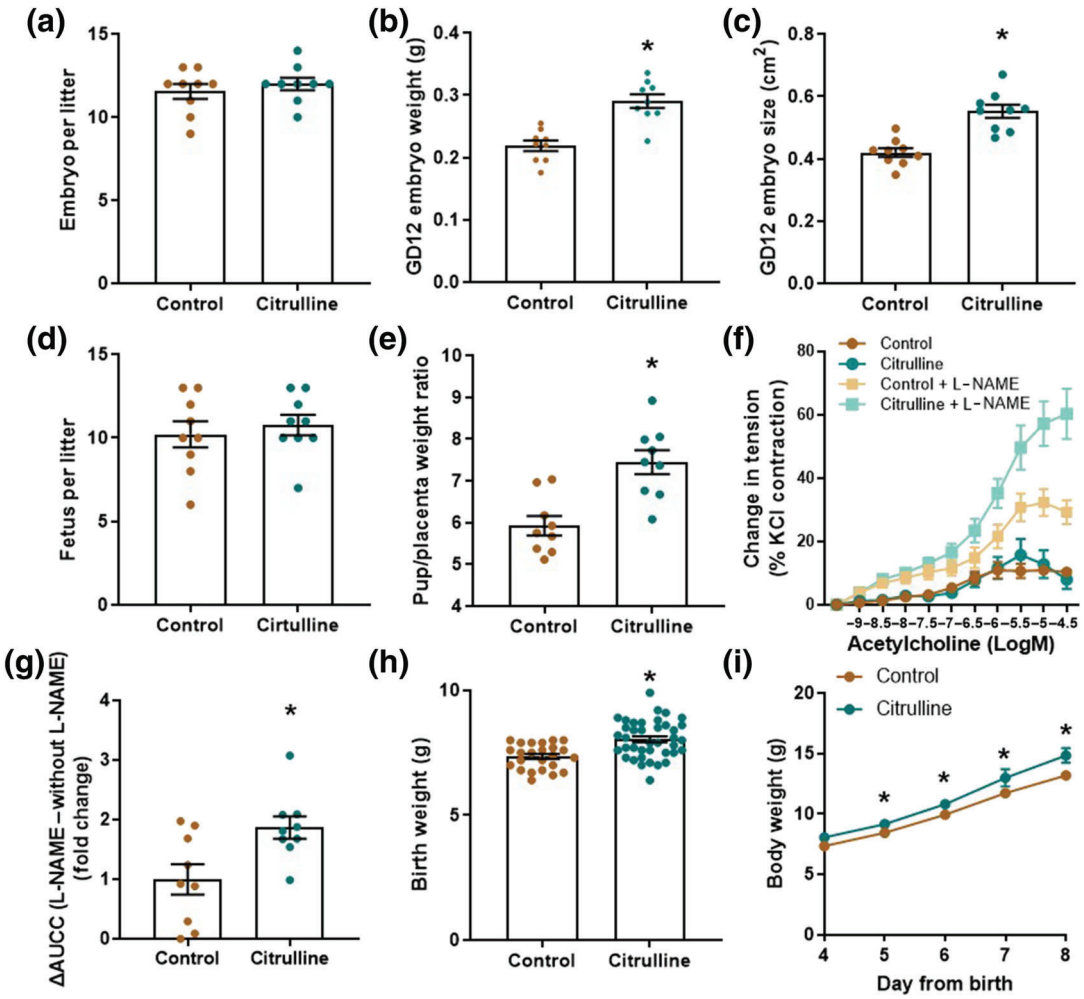

FIGURE 2 Citrulline improves placental insufficiency and improves fetal growth in DSSR. L-Citrulline (2.5 g·L^{-1} in drinking water) was administered from the day of mating to the end of the experiments. At GD12, the number of embryos per litter (a) and the weight (b) and size (c) of the embryos were measured. At GD 21, the number of fetuses per litter (d) and the ratio of pup-to-placenta weight (e) were measured. The vascular responsiveness of the GD 21 umbilical vein was measured using wire myography. Preparations were incubated for 30 min with or without L-NAME (10^{-4} M). Preparations were then exposed to increasing concentrations of acetylcholine to induce contraction (f). L-NAME-induced contraction was analysed by calculating the difference between area under the contraction curve (ΔAUCC) (g). The birth weight of the offspring was measured (h), and the body weight of the pups was measured from the age of day 4 to 8 (i). Data are presented as means ± SEM (f, i) or with individual values (a-e, g, h). *P < 0.05, significantly different from control

contraction of the umbilical vein (Figure 2f), which is consistent with previous reports (Haugen, 2015). In the presence of the NOS inhibitor L-NAME, acetylcholine-induced contraction was much larger in the umbilical vein from citrulline-treated DSSR (Figure 2g), indicative of improved NO production by citrulline. The L-NAME-induced contraction was used as an indicator of endogenous NO production that counterbalances vasoconstriction (De Silva et al., 2011). The birth weight of the offspring was significantly increased in the citrulline-treated DSSR (Figure 2h,i).

3.3 | Citrulline improves endothelial function in pregnant DSSR

Serum citrulline and arginine levels were significantly increased by citrulline treatment in pregnant DSSR (Figure S1). EPR spectra of NO-Fe $(DETC)_2$ were obtained and used to assess the in vitro NO production in the aorta of DSSR (Figure 3a). Our results suggested a significantly higher NO production in the aorta of citrulline-treated DSSR compared with control (Figure 3b). Also, citrulline supplementation augmented endothelial NO signalling in DSSR. The total protein expression of both **endothelial nitric oxide synthase** (eNOS) and its key downstream target, the vasodilator-stimulated phosphoprotein (VASP), was significantly up-regulated in the citrulline-treated DSSR (Figure 3c). Relative phosphorylation of VASP was also significantly increased in the citrulline-treated DSSR.

Citrulline supplementation significantly improved the vascular function of the second order mesenteric arteries in pregnant DSSR. Endothelium-dependent, acetylcholine-mediated vasorelaxation was increased in citrulline-treated DSSR (Figure 3d). Endothelium-derived hyperpolarizing factor (EDHF) was measured as the NO/prostaglandin-independent component of endothelium-dependent relaxation. In the presence of both L-NAME and indomethacin, citrulline significantly improved the EDHF-mediated relaxation of mesenteric arteries (Figure 3e). Next, we examined the expression of genes related to EDH in the mesenteric arteries. Citrulline treatment significantly promoted the expression of *Trpv1*, ***Trpv4*** and **Kcnn4** while reducing the expression of **Kcnn3.** Note also that expression of two connexin genes, **Gja4 for Cx37** and **Gja5 for Cx40**, were unchanged by citrulline (Figure 3f).

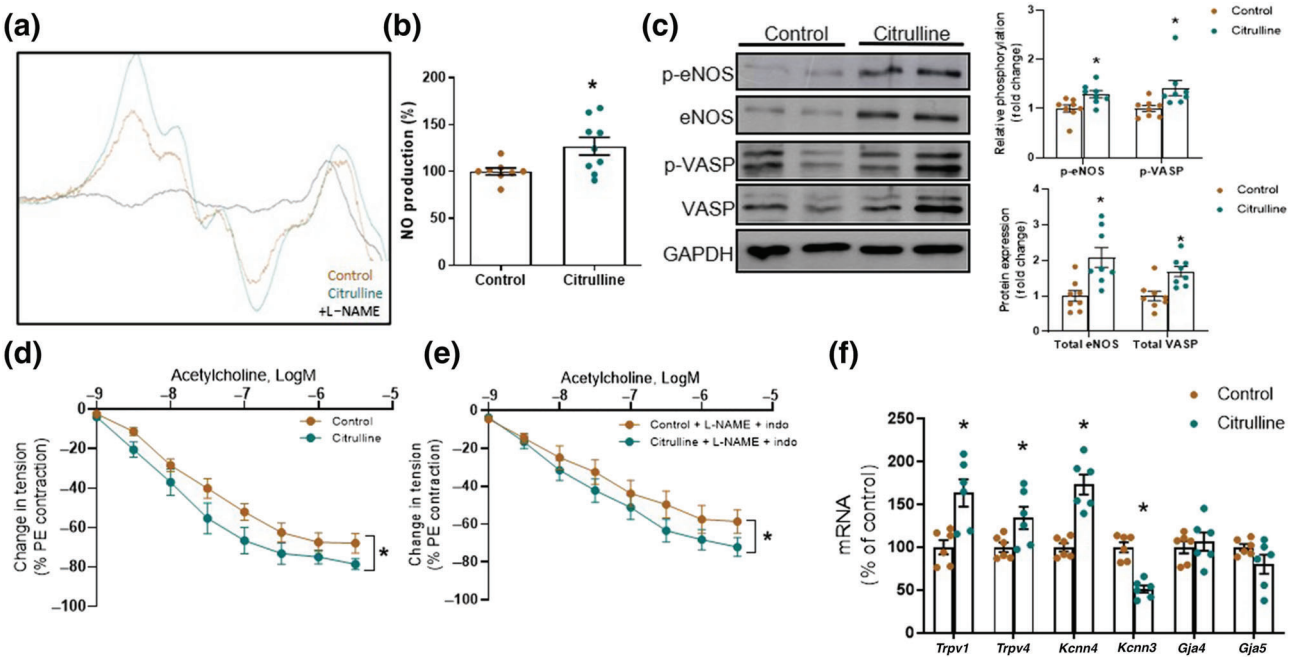

FIGURE 3 Citrulline improves endothelial function in pregnant DSSR. L-Citrulline (2.5 $g{\cdot}L^{-1}$ in drinking water) was administered from the day of mating to the end of the experiments. At GD12, production of NO in the aorta of the pregnant rats was measured by electron paramagnetic resonance spectrometry (a), and the relative percentage of the NO production was calculated (b). Protein expression of phosphorylated and total eNOS, phosphorylated, and total VASP in the aorta of the pregnant rats was analysed by Western blotting. GAPDH was used as the internal control. Total protein level was normalized to internal control. Relative phosphorylation was normalized to total protein level (c). The vascular responsiveness of the second-order mesenteric artery of the pregnant rats was studied using wire myography. Preparations were pre-contracted with phenylephrine (PE). Basal endothelium-dependent relaxation was assessed by exposing the preparations to increasing concentrations of acetylcholine (d). Endothelium-derived hyperpolarizing factor (EDHF) was assessed by exposing the preparations to acetylcholine in the presence of L-NAME (10^{-4} M) and indomethacin (indo, 10^{-5} M) (e). Control = 11 vessels, citrulline = 14 vessels. The expression of genes related to endothelium-dependent hyperpolarization (*Trpv1*, TRPV1; *Trpv4*, TRPV4; *Kcnn4*, $K_{Ca}3.1$; *Kcnn3*, $K_{Ca}2.3$; *Gja4*, CX37; *Gja5*, CX40) in the mesenteric arteries was measured by quantitative PCR (f). Data are presented as means ± SEM (d, e) or with individual values (b, c, f). *$P < 0.05$, significantly different from control

3.4 | Citrulline ameliorates fibrosis and promotes angiogenesis in the placenta

Histological features of placentas in preeclamptic patients are characterized by chronic inflammation and fibrosis, while placental fibrosis can be triggered by TGF-β1 signalling (Ohmaru-Nakanishi et al., 2018). Therefore, we examined the histological features of placentas in DSSR. Citrulline ameliorated placental fibrosis in DSSR. Collagen deposition in the placenta, as indicated by the blue colour stained by Masson's' trichome, was significantly reduced by citrulline (Figure 4a, b). No significant changes in TGF-β1 expression was found. But the expression of a fibrotic marker, **metalloproteinase 9** (MMP9), was down-regulated in the placentas of citrulline-treated DSSR (Figure 4c).

Immunostaining for CD31, a marker of endothelial cells, was used to examine the placental vascularization. The results showed that the microvessel count was increased in the citrulline-treated DSSR placenta, compared with control (Figure 4d). Gene expression of anti-angiogenic factors was reduced, while the gene expression of angiogenic factors was increased in the placenta of citrulline-treated DSSR compared to control (Figure 4e). Protein expression of CD31 was also increased in the citrulline-treated DSSR compared with control (Figure 4f). In addition, the protein expression of PlGF and VEGF was up-regulated in the citrulline-treated DSSR (Figure 4g). These results suggested citrulline could improve angiogenesis in the placentas of DSSR.

3.5 | Citrulline ameliorates placental senescence during pregnancy in DSSR

Placental senescence has been associated with preeclampsia (Sukenik-Halevy et al., 2016). **Liver kinase B1** (LKB1) is a serine/threonine kinase that is highly expressed in senescent cells. LKB1 induces vascular senescence (Zu et al., 2010) and inhibits

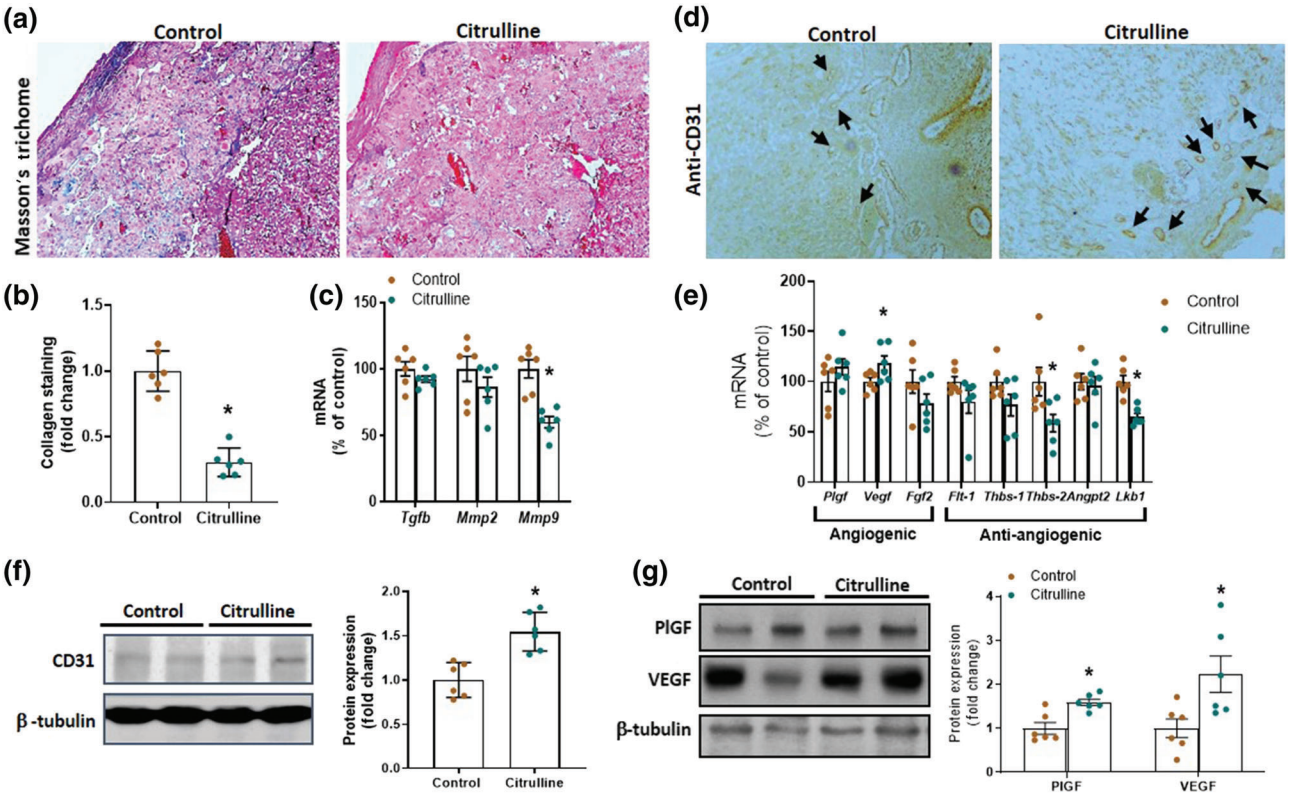

FIGURE 4 Citrulline ameliorates fibrosis and promotes angiogenesis in the placenta. L-Citrulline (2.5 g·L^{-1} in drinking water) was administered from the day of mating to the end of the experiments. At GD21, the placentas were fixed in formalin and embedded in paraffin. Placenta sections were stained using Masson's trichome staining kit, and the blue colour indicates positive stain for collagen. Magnification: 100× (a). Collagen staining was quantified using ImageJ software (b). $n = 6$ animals per group. The expression of remodelling genes, for TGF-β (*Tgfb*) and MMP 2 and 9 (*Mmp2* and *Mmp9*), in the placenta was analysed by quantitative PCR (c). The expression of endothelial cell marker CD31 was examined by immunohistochemistry (IHC) staining using anti-CD31 antibody. Magnification: 100× (d). Black arrows indicate small vessels with positive stain. The expression of angiogenic (*Plgf*, placental growth factor; *Vegf*, vascular endothelial growth factor; *Fgf2*, fibroblast growth factor) and anti-angiogenic (*Flt-1*, fms-like tyrosine kinase 1; *Thbs*, thrombospondin; *Angpt2*, angiopoietin 2; *Lkb1*, liver kinase b1) genes in the placenta was measured by quantitative PCR (e). The protein expression of CD31 (f), placental growth factor (PlGF), and vascular endothelial growth factor (VEGF) (g) in the placenta was measured by Western blotting. β-tubulin was used as internal control. Data are presented as means ± SEM, with individual values. *$P < 0.05$, significantly different from control

VEGF-induced angiogenesis (Okon et al., 2014). In the placentas of citrulline-treated DSSR, LKB1 was down-regulated (Figure 4e). Therefore, we further investigated the placental senescence in DSSR.

Gene expression of the senescence marker p16 was significantly down-regulated in the placentas of citrulline-treated DSSR, while gene expression of p21 and p53 also has a trend of reduction (Figure 5a). Next, we examined whether the placental senescence was facilitated by circulating factors in the serum. Endothelial cells were incubated with or without the serum of pregnant DSSR. The results suggested that endothelial cells incubated with serum from citrulline-treated DSSR had lower numbers of β-gal-positive cells, than those incubated with control serum (Figure 5b). Gene expression of p53 in endothelial cells was significantly up-regulated by control DSSR serum whereas serum from citrulline-treated DSSR reduced p53 expression, relative to control DSSR serum (Figure 5c). These suggested that citrulline supplementation may ameliorate placental senescence in DSSR.

3.6 | Citrulline regulates differential gene expressions in DSSR placentas

High serum levels of TLR4 and NF-κB have been found in patients with preeclampsia, and have been proposed as biomarkers for predicting preeclampsia (Litang et al., 2017). Therefore, we studied the expression of genes involved in TLR4/NF-κB signalling in the placenta of DSSR. Expression of the genes *Tlr4* and its downstream molecule myeloid differentiation factor 88 (*Myd88*), as well as the subunits of NF-kB (*p65* and *p50*) in the DSSR placentas, were significantly reduced by citrulline supplementation (Figure 6a). Gene expression of downstream inflammatory markers of NF-κB, including **inducible nitric oxide synthase** (iNOS), vascular cell adhesion protein 1 (**VCAM1**), and intercellular adhesion molecule 1 (**ICAM-1**), was significantly down-regulated in the placentas of citrulline-treated DSSR (Figure 6b).

Placental gene expression of TNF-α and HIF-1α was down-regulated by citrulline supplementation. Citrulline up-regulated the gene expression of **SIRT1**, which is an important stress-response protein that modulates feto-placental vascular development (Pham et al., 2018) (Figure 6c). The gene expression of enzymes related to lipid metabolism, including paraoxonase 2 (*Pon2*), **protein kinase AMP-activated non-catalytic subunit γ2** (*Prkag2*), and **phospholipase A_2** (*Pla2*), was recently reported to be differentially expressed in the placentas of Sprague–Dawley (SD) rats and DSSR (Maeda et al., 2019). Interestingly, the expression of *Pon2* and *Pla2* was significantly up-regulated in citrulline-treated DSSR (Figure 6d).

In endothelial cell culture, incubation with the serum of control DSSR led to the up-regulation of components of the TLR4 signalling pathway (TLR4, MYD88, and p65) in vitro. These effects were largely normalized by the serum from citrulline-treated DSSR. Moreover, incubation with the serum of citrulline-treated DSSR significantly promoted the expression of *Pon2* (Figure 6e). These results suggested that citrulline may regulate differential gene expression in DSSR placenta.

4 | DISCUSSION

The present study demonstrates the protective effects of L-citrulline supplementation in an animal model of preeclampsia. Citrulline

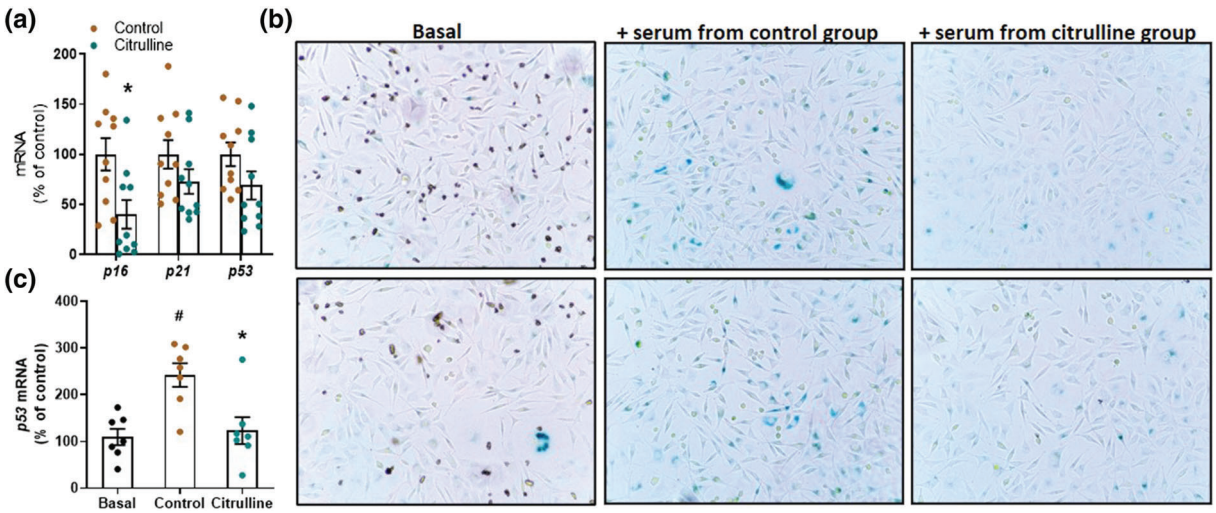

FIGURE 5 Citrulline ameliorates placenta senescence in DSSR. L-Citrulline (2.5 g·L^{-1} in drinking water) was administered from the day of mating to the end of the experiments. At GD21, the gene expression of senescence markers p16, p21, and p53 in the rat placenta was measured by qPCR (a). Human umbilical vein endothelial cells-derived EA.hy926 cells were incubated either with the normal growth medium (basal), or with basal medium supplemented with serum (4%) from control DSSR or citrulline-treated DSSR for 72 h. Endothelial senescence was examined by β-galactosidase senescence detection kit (Abcam). The development of the bluish-green colour indicates senescent cells. Magnification: 200× (b). Gene expression of p53 in the EA.hy926 incubated with the serum of either control or citrulline-treated rats was examined by qPCR (c). Data are presented as means ± SEM with individual values. $^{*}P < 0.05$, significantly different from control; $^{\#}P < 0.05$, significantly different from basal levels

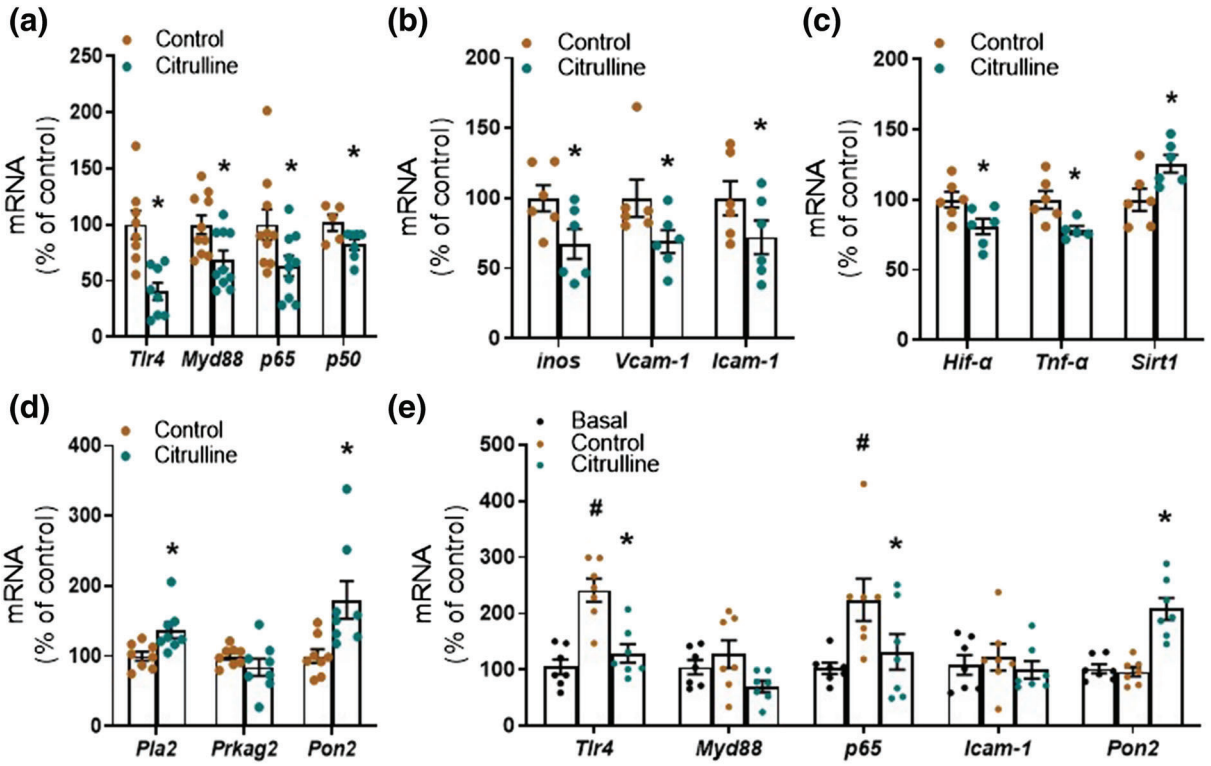

FIGURE 6 Citrulline regulates expression of different genes in DSSR placenta. L-Citrulline (2.5 g·L^{-1} in drinking water) was administered from the day of mating to the end of the experiments. Placentas from GD21 rats were collected for analysis. Expression of genes in the TLR4 pathway including *Tlr4* and its downstream component MyD88 (*Myd88*) and subunits of NF-κB, *p65* and *p50*, in the DSSR placenta was measured by quantitative PCR (a). Gene expression of downstream inflammation markers of NF-κB including *inos*, *Vcam-1* and *Icam-1* in DSSR placenta was measured by quantitative PCR (b). Gene expression of *Hif-α*, *Tnf-α* and *Sirt1* in DSSR placenta was measured by quantitative PCR (c). Expression of genes related to lipid metabolism, including protein kinase AMP-activated non-catalytic subunit gamma 2 (*Prkag2*), *Pla2*, and *Pon2* in DSSR placenta was measured by quantitative PCR (d). Human umbilical vein endothelial cells-derived EA.hy926 cells were incubated with the normal growth medium (basal) or basal medium supplemented with serum (4%) from control or citrulline-treated DSSR for 72 h. Gene expression in the EA.hy926 was examined by qPCR (e). Data are presented as means ± SEM with individual values. $^{*}P < 0.05$, significantly different from control. $^{\#}P < 0.05$, significantly different from basal

supplementation in the drinking water of pregnant DSSR leads to (i) reduction of maternal blood pressure and markers of preeclampsia; (ii) improvement of maternal endothelial function; (iii) amelioration of placental fibrosis and senescence; (iv) promotion of placenta angiogenesis; and (v) improvement of fetal growth.

4.1 | Significance of this study

Beneficial effects of citrulline supplementation on cardiometabolic health has been extensively studied (Rashid et al., 2020). However, the effect on preeclampsia and the long-term effects to fetal outcomes remain unclear. There were 34 results using the keyword "citrulline + preeclampsia" found in the PubMed search engine at the time we finished this study. However, most of them studied the correlation between serum levels of citrulline and preeclampsia, and none of them studied the direct effect of citrulline supplementation in preeclampsia. Only shortly after we had completed this study, a report was published online ahead of print, which demonstrated that citrulline improves perinatal and postpartum maternal vascular function in a preeclampsia-like genetically modified mouse model (Gemmel et al., 2021). Compatible with our findings, Gemmel et al. suggest that citrulline has beneficial effects on maternal vascular health in C1q$^{-/-}$ mice, attributable to NO signalling. Nevertheless, the effect of citrulline in placental development and gene expression remains unclear. In our present study, we demonstrated that a number of mechanisms could be involved in the beneficial effects of citrulline in preeclampsia, including (i) maternal vascular function, (ii) improved placental function, and (iii) a direct effect on the fetus.

4.2 | Maternal vascular function

Production of NO is increased during normal pregnancy and decreases in preeclampsia (Suzuki et al., 2009). Reduction in eNOS/NO exacerbates sFlt-1-associated preeclampsia-like phenotype in mice (Li et al., 2012). NOS inhibition by L-NAME perfusion has been used in rodent models to mimic the preeclampsia-like phenotype (Lemery Magnin et al., 2018). These suggest that NO plays a crucial role in maintaining a healthy pregnancy. Vascular dysfunction in

preeclampsia can be presented as augmented arterial stiffness and reduced vasodilatation (Enkhmaa et al., 2016). Our data suggest that citrulline supplementation can improve maternal vascular function of both large conduit arteries and small resistance arteries. Endothelium-dependent vasodilation of large conduit arteries is mainly mediated by eNOS-derived NO (Shimokawa, 2014). In large arteries, enhanced eNOS expression and NO production (Figure 3a–c) protects against abnormal constrictions and against atherosclerosis (Takaki et al., 2008; Vanhoutte et al., 2017). In contrast, EDHF is the key player in small resistance arteries (Shimokawa, 2014). Down-regulation of endothelial calcium-dependent potassium (K_{Ca}) channels, is associated with impaired EDHF-mediated vasorelaxations in patients with preeclampsia (Choi et al., 2013). Down-regulation of TRPV1 and TRPV4 expression is associated with the hypertension in DSSR (Gao & Wang, 2010; Wang & Wang, 2006). Thus, the enhanced expression of TRPV1 and TRPV4 (Figure 3f) by citrulline supplementation may represent a mechanism of improved vasodilation mediated by EDHF (Figure 3e). Importantly, the EDHF component of vasodilation in the mesenteric artery is reduced by about 50% in the global eNOS-knockout mice (Takaki et al., 2008), indicating a substantial contribution of eNOS to EDHF action. Therefore, the enhanced eNOS expression is likely to be the second mechanism contributing to the improvement of EDHF-mediated vasodilation in small resistance arteries in citrulline-treated DSSR. This eNOS-mediated EDHF (hydrogen peroxide) production is resistant to NOS inhibitors (Takaki et al., 2008; Vanhoutte et al., 2017). Collectively, the improved vasorelaxation in the resistance arteries may be an explanation for the reduced blood pressure in the pregnant DSSR.

4.3 | Placental inflammation and fibrosis

Poor placentation leads to the early onset of preeclampsia. Preeclampsia can occur in patients with hydatidiform moles (Acosta-Sison, 1956). NO participates in the process of placentation, placental angiogenesis and endothelial function in the placenta (Krause et al., 2011). Recent studies have suggested the importance of NO signalling in modulating the angiogenic factors including PlGF, VEGF, angiopoietin (*Angpt*), thrombospondin (*Thbs*), and anti-angiogenic factors like sFlt1 (Failla et al., 2018). The imbalance of these factors is associated with placental insufficiency and preeclampsia (LeBlanc & Kelm, 2017). Citrulline may improve placental function and fetal growth by promoting NO synthesis. Our data suggest a promotion of placental angiogenesis in the placentas of DSSR, which is associated with an up-regulation of angiogenic factors and a down-regulation of anti-angiogenic factor.

As reported in another study, using a rat model of intrauterine growth restriction induced by maternal dietary protein restriction (Bourdon et al., 2016), we also observed no significant change in placental eNOS gene expression after citrulline supplementation (Figure S2). Opposite to their finding, we observed a down-regulation of placental iNOS, which could be due to the inhibition of NF-κB-mediated transcription of iNOS in pregnant DSSR by citrulline. NF-κB-mediated inflammatory cytokines and oxidative stress may exert a pronounced effect in the fetus. Blunted up-regulation of iNOS has been proposed to attenuate preeclampsia (Amaral et al., 2013). Citrulline can down-regulate the expression of NF-κB and its downstream inflammatory markers in the placentas of DSSR.

TGF-β-mediated fibrosis is one of the most prominent pathological features of preeclamptic placentas (Ohmaru-Nakanishi et al., 2018). The DSSRs have increased placental hypoxia, which is a known factor that leads to fibrosis (Higgins et al., 2007). Our results show that citrulline can reduce placental fibrosis in DSSR, which is associated with the down-regulation of HIF-1α. A significant up-regulation of TLR4 signalling pathway in the placentas of DSSR compared with SD rats has been reported (Ishimwe et al., 2019). The activation of TLR4 signalling pathway in the placenta promotes pro-inflammatory cytokines production through the up-regulation of several transcription factors, including NF-κB, which can induce inflammatory responses and placental cytotrophoblast apoptosis (Chen et al., 2015). Inhibition of HIF-1α-mediated TLR4 activation can attenuate apoptosis and promote placental angiogenesis during severe preeclampsia (Zhao et al., 2019). Our results suggest that citrulline supplementation improves placental phenotype in DSSR, at least partly, by down-regulating HIF-1α-mediated TLR4 activation in the placentas.

4.4 | Placental senescence

Premature aging of the placenta has been recently associated with pregnancy complications including preeclampsia and intrauterine growth restriction (Sukenik-Halevy et al., 2016). Preeclamptic women exhibit increased placental senescence, compared with normal pregnant women (Sukenik-Halevy et al., 2016). Protein and gene expression of senescence markers (p16, p21, and p53) are up-regulated in the placenta from preeclamptic patients (Londero et al., 2016; Yang et al., 2015). Chronic low-grade inflammation increased placental oxidative stress and endoplasmic reticulum stress can facilitate placental senescence (Redman & Sargent, 2009). DSSR has increased placental hypoxia.

NO bioavailability is reduced in senescent cells, while increasing NO bioavailability can activate telomerase and delay endothelial cell senescence (Hayashi et al., 2008). Our results demonstrate that L-citrulline can also attenuate placental senescence in preeclampsia, possibly by restoring NO levels. The serine/threonine kinase, **LKB1**, is highly expressed in senescent cells and can cause vascular senescence (Zu et al., 2010) and inhibit VEGF-induced angiogenesis (Okon et al., 2014). LKB1 is also an upstream inducer **of AMP-activated protein kinase** (AMPK) and amino acid-transporter required for **mammalian target of rapamycin complex 1** (mTORC1) activity, which are up-regulated in preeclampsia (Huang et al., 2020). However, the detailed involvement of LKB1 in preeclampsia remains unclear. The reduction of LKB1 expression in the placentas of citrulline-treat DSSR may be attributed to the increased expression of placental **SIRT1**. Indeed, SIRT1 is an important regulator of LKB1 level (Bai

et al., 2016). Reduced expression and activity of SIRT1 in endothelial cells leads to the accumulation of acetylated LKB1, which cause senescence (Bai et al., 2016). Therefore, citrulline may attenuate placental senescence in preeclampsia via activating SIRT1-mediated down-regulation of LKB1. Further studies are warranted to explore the detailed mechanism of placental senescence in which may provide novel therapeutic strategies for preeclampsia.

4.5 | Lipid metabolism

Apart from the beneficial effects in improving vascular function and blood flow, citrulline supplementation may affect the efficiency of nutrient transfer in placentas. Consistent with another study (Fowden et al., 2008), we observed an increased the fetal-to-placental weight ratio, an index of placental efficiency, after citrulline supplementation. This suggests that citrulline can improve the placental structural and functional adaptations to maintain fetal growth.

Lipids and fatty acids supplied through the placenta are important for fetal growth. However, excessive flux of lipids could promote oxidative stress and inflammatory cascades, contributing to preeclampsia (Tenorio et al., 2019). A recent temporal transcriptomic analysis study in DSSR has identified a cluster of aberrantly regulated genes involved in lipid and fatty acid metabolism, including the apolipoprotein family, *Pon2*, *Prkag2* and *Pla2* (Maeda et al., 2019). PON2 is an important antioxidant enzyme that is associated with lipid metabolism and insulin sensitivity (Manco et al., 2021). PLA_2 is involved in the regulation of fatty acid and phospholipid metabolism while Prkag2 is involved in glucose and lipid metabolism (Steiger et al., 2019). In the present study, we report an up-regulation of *Pon2* and *Pla2* in the placentas of citrulline-treated DSSR. Moreover, the SIRT1-**PPARγ** axis is proposed to be a key player in trophoblast differentiation and placental development (Pham et al., 2018). The down-regulation of SIRT1 expression in the placenta might be responsible for the increased NF-κB transcriptional activity and reduction of PPARγ (Lappas et al., 2011). These results suggest an important link between placental lipid metabolism and preeclampsia, which warrants further studies.

4.6 | Limitations of the study

Preeclampsia is a spontaneous complication that exclusively occurs in pregnant humans and certain apes. There is currently no animal model which perfectly mimics human preeclampsia. All the animal preeclampsia models available have limitations due to their differences from pregnant women, which represents a challenge in this area of study (Marshall et al., 2018), and we realise that pregnant DSSR may not fully replicate the human condition. Therefore, the results of the present study cannot be completely translated to the human situation. However, the DSSR phenotype is consistent with many of the characteristics observed in human superimposed preeclampsia, including exacerbation of hypertension during pregnancy, proteinuria, placenta hypoxia, enhanced placenta production of TNF-α, as well as in increased plasma sFlt-1 (Gillis et al., 2015). Moreover, recent studies using DSSR have demonstrated the similarity of the preeclamptic phenotype to that of human preeclampsia, including changes in in lipid and fatty acid metabolism (Maeda et al., 2019), cerebral oedema, and blood–brain barrier disruption (Maeda et al., 2021), gut microbial remodelling (Ishimwe et al., 2021), and immunological changes (Taylor et al., 2021). Therefore, the DSSR model may represent an important animal model for studying the pathomechanisms of preeclampsia and for developing therapeutic interventions.

In the present study, we have shown that maternal citrulline treatment can improve preeclampsia pathologies and fetal growth. Nevertheless, we do not know the exact molecular mechanisms of citrulline yet. Our results have shown that the incubation of serum from citrulline-DSSR can ameliorate placental senescence and regulate the expression of different genes in endothelial cells in vitro. This suggests the possible contribution of citrulline-related metabolites. NO may mediate transcriptional regulation of histone-modifying enzymes through the formation of *S*-nitrosothiols or iron nitrosyl complexes (Socco et al., 2017). Metabolites of citrulline and arginine, such as polyamines, are required in several stages of pregnancy (Aye et al., 2021). Reductions in polyamine bioavailability in pregnant rodents have been associated with abnormal placentation and fetal growth restriction (Hsu & Tain, 2019). Recent study also reveals the epigenetic effect of placental polyamines by regulating acetyl-CoA level and histone acetylation (Aye et al., 2021). Thus, citrulline metabolites may contribute to the effects observed in the present study. Another possible mechanism is a direct stimulation of protein synthesis to improve fetal growth (Le Plenier et al., 2017), probably by activating the phosphorylation of proteins in the mTOR signalling pathway (Jourdan et al., 2015).

Another limitation is that we did not examine the reprogramming effect of maternal citrulline supplementation (i.e., the disease risk of the offspring at adult age). Maternal treatment with citrulline of the spontaneously hypertensive rats ameliorated the development of hypertension in the offspring (Koeners et al., 2007). We have observed an improvement of fetal growth in the DSSR. Thus, a reprogramming effect of maternal citrulline treatment is conceivable in the DSSR. Nevertheless, this was not investigated, because it is out of scope of this study. This cardiovascular and metabolic disease risk of the offspring from citrulline-treated mothers should be further addressed in future studies.

Finally, a normal control group was not included in our study. In the previous study, the DSSR model has been compared with the Sprague–Dawley rats (with normal pregnancy) and the spontaneously hypertensive rats (without a preeclamptic phenotype despite preexisting hypertension). The comparison has provided evidence of preeclampsia phenotypes in the DSSR model (Gillis et al., 2015). In the present study, we have observed amelioration of the preeclampsia phenotypes in citrulline-treated DSSR. Nevertheless, without a normal control group, we cannot calculate the exact extent of the therapeutic effects of citrulline and do not know whether citrulline treatment leads to complete normalization.

In conclusion, this study shows that L-citrulline supplementation in a rat model of superimposed preeclampsia can reduce maternal hypertension and improve placentation and fetal growth. L-Citrulline could be a potent and safe therapeutic strategy for preeclampsia that benefits both the mother and the fetus.

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AUTHOR CONTRIBUTIONS

NX and HL designed the study. AWCM, YZ, UDPL, GR, AW, and AH performed the experiments and analysed data. AWCM wrote the manuscript. AD, TM, EC, NX, and HL critically reviewed and edited the manuscript. All authors agreed to its publication.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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