Fumaric acid and succinic acid treat gestational hypertension by downregulating the expression of KCNMB1 and TET1

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Abstract. The present study hypothesized that fumaric acid and succinic acid may exhibit therapeutic effects on gestational hypertension. During pregnancy, estrogen upregulates ten-eleven translocation 1 (TET1) expression, which subsequently increases calcium-activated potassium channel subunit β1 (KCNMB1) expression. KCNMB1 is associated with hypertension. Fumaric acid and succinic acid are understood to inhibit TET. Therefore, the present study investigated whether fumaric acid and succinic acid exhibit therapeutic effects on gestational hypertension and whether these effects are mediated by TET1 and KCNMB1. Nω-Nitro-L-arginine methyl ester hydrochloride was injected into rats to establish a gestational hypertension model. Dimethyl fumarate (DMF) and succinic acid were administrated into rats to treat gestational hypertension. Rats were divided into five groups: i) Control; ii) model; iii) DMF; iv) succinic acid; and v) DMF + succinic acid. Blood pressure was monitored by a noninvasive meter and urinary protein was determined using a urinary protein kit. Placenta pathology was examined by hematoxylin-eosin staining. Compared with the control group, urinary protein and blood pressure in the model group increased significantly. The placental cells in the control group were arranged orderly. However, in the model group, decidual cellular edema of placenta and vacuolar degeneration were observed, and the intervascular membrane was markedly thicker with plenty of fibrin deposition. These results indicate successful establishment of a gestational hypertension model. However, compared with the model group, urinary protein, blood pressure, edema, vacuoles and fibrin deposition were markedly reduced in the DMF, succinic acid and DMF + succinic acid groups. mRNA and protein levels of TET1 and KCNMB1 in placenta were evaluated by immunohistochemical analysis, reverse transcription-quantitative polymerase chain reaction and western blotting. The TET1 and KCNMB1 levels in the model group were markedly increased compared with those in the control group. However, compared with the model group, the expression levels were markedly downregulated in the DMF, succinic acid and DMF + succinic acid groups. In conclusion, fumaric acid and succinic acid may treat gestational hypertension by downregulating the expression of KCNMB1 and TET1.

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Introduction

Gestational hypertension is a common obstetric disease, occurring in 5-10% of pregnancies (1). Maternal death induced by gestational hypertension accounts for 10-16% of all pregnancy-associated mortalities and gestational hypertension is the second leading cause of maternal death in China (1). Hypertension, proteinuria and edema are the main symptoms of this condition. Gestational hypertension may be induced by maternal, placental and fetal factors, such as abnormal invasion of trophoblasts, abnormal immune regulation, endothelial cell damage, genetic factors and nutritional factors (2). No single factor can explain the complex etiology and mechanism of gestational hypertension (3). In the treatment of gestational hypertension, blood pressure must be lowered and severe preeclampsia and eclampsia must be prevented (4).

Metoprolol succinate is a selective β1 receptor blocker. The dose required for its action on cardiac β1 receptors is lower than that required for its action on peripheral blood vessels and bronchi β2 receptors (5). Metoprolol succinate doesn't activate β receptors on membranes. Metoprolol succinate can attenuate the effect of catecholamines associated with physiological and psychological loads, and reduce the heart rate, cardiac output and blood pressure (5). Bisoprolol fumarate is also a highly selective β1 adrenoceptor antagonist, without intrinsic sympathomimetic and membrane stabilization activities. Bisoprolol fumarate has a high affinity for β1 receptors of bronchi and vascular smooth muscle, which leads to vasodilation and lower blood pressure (5). Metoprolol succinate and bisoprolol fumarate are widely used in the treatment of hypertension (5). In addition, during pregnancy, estrogen upregulates ten-eleven translocation 1 (TET1) expression in uterine arteries, which activates demethylation and subsequently increases

calcium-activated potassium channel subunit β1 (KCNMB1) expression (6). KCNMB1 is associated with hypertension (7). Fumaric acid and succinic acid are known to inhibit TET (8). Therefore, the present study hypothesized that fumaric acid and succinic acid may exhibit therapeutic effects on gestational hypertension, and the present study was performed for preliminary confirmation.

The TET1 gene is a research hotspot at present for its special role in DNA methylation (9). TET1 is a hydroxymethylase, originally discovered as a fusion protein of histone H3K4 methyltransferase (10). TET1 can bind to the CpG region in the genome, which hinders catalytic activity of DNA methyltransferase and activates gene transcription (11). In addition, TET1 can recruit polycomb repressive complex 2 to the promoter region of a target gene, which ultimately suppressed the expression of the target gene (12). The TET protein family participates in the whole embryonic development process. When TET1 and TET3 are knocked-out at the same time, the transcriptome diversity increases during early embryonic development (13). Growth defects, increased mortality and developmental retardation are identified in the offspring of paternal mice following TET1-knockdown (14). BKCa channels are involved in the regulation of cellular functions, including neurotransmitter release, hormone secretion, heart rate, vascular stress resistance and smooth muscle tension, for their multiple regulatory characteristics. BKCa is composed of an ion-mediated α subunit and different β subunits. The β1 subunit is predominantly expressed in smooth muscle cells and it affects the behavior of these cells (15). Mutations in the KCNMBl gene encoding the β1 subunit are associated with heart rate variability and baroreflex function (16). Variation of the KCNMBl gene loci decreases arterial impedance (17). KCNMBl may serve an important role in the development and progression of hypertension (18).

Therefore, the present study established a rat gestational hypertension model and investigated whether fumaric acid and succinic acid exhibit therapeutic effects on gestational hypertension and whether these effects are mediated by TET1 and KCNMBl.

Materials and methods

Materials and animals. Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME; cat. no. 51298-65.5) and dimethyl fumarate (DMF; cat. no. C1821194) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Succinic acid (cat. no. ZN1113EA14) was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. The rat urinary protein kit (cat. no. CO35-2) was obtained from Nanjing Jiancheng Bioengineering Institute. The bicinchoninic acid (BCA) kit (cat. no. CW0014), diaminobenzidine (DAB) kit (cat. no. CW0125), TRIzol reagent (cat. no. CW0580), Ultrapure RNA extraction kit (cat. no. CW0581), HiFiScript cDNA synthesis kit (cat. no. CW2569) and ULtraSYBR Mixture (cat. no. CW0957) were purchased from CoWin Biosciences Co., Ltd. (CWBIO). RIPA lysis buffer (cat. no. C1053) was purchased from Applygen Technologies, Inc. SuperSignal® west pico chemiluminescent substrate (cat. no. RJ239676) was obtained from Thermo Fisher Scientific, Inc. The polyvinylidene fluoride (PVDF) membrane (cat. no. IPVH00010) was obtained from EMD Millipore. Mouse anti-GAPDH monoclonal antibody (cat. no. TA-08; 1:2,000), horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (H+L) (cat. no. ZB-2305; 1:2,000) and HRP conjugated goat anti-rabbit IgG (H+L) (cat. no. ZB-2301; 1:2,000) were purchased from OriGene Technologies, Inc. Rabbit anti-TET1 polyclonal antibody (cat. no. DF6428; 1:500) and rabbit anti-KCNMB1 polyclonal antibody (cat. no. DF-9301; 1:1,000) were purchased from Affinity Biosciences. Rabbit anti-KCNMB1 polyclonal antibody (cat, no. bs-7689R; 1:1,000) was obtained from BIOSS.

In total, 35 female (three months-old, 250-280 g) and 20 male (three months-old, 400-500 g) Sprague-Dawley rats were obtained from the Hunan SJA Laboratory Animal Co., Ltd. [license no. SCXK(Xiang)2016-0002]. All rats were provided with free access to food and water and kept in conditions of 22-25˚C under a 12-h light/dark cycle. During the experimental phase, animal well-being was monitored by individual ventilated caging system (SmartRack, BioZone). Unpregnant rats and male rats after mating was completed and the fetuses after birth were fed normally and sacrificed at the end of the whole experiment. The experiments did not affect normal activity and eating behavior of rats. Rats were sacrificed after anesthesia. The study protocol was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Guiyang University of Chinese Medicine.

Gestational hypertension model establishment. Overnight, female and male rats were placed in a cage at a ratio of 2:1 to induce pregnancy. Formation of vaginal plug was observed the next morning. A total of 30 female rats were impregnated. Rats with vaginal plug were immediately placed in separate cages and day 0 of pregnancy was record. Blood pressure was measured on day 9 of pregnancy using a constant temperature noninvasive blood pressure meter (XH200; Beijing Zhongshidichuang Co., Ltd.). The rats were then randomly grouped (n=6). Drugs were administrated by gavage at 10 ml/kg q.o.d, from day 10 of pregnancy to the birth of the fetus. Concentrations of DMF and succinic acid were 2.5 and 40 mg/ml, respectively. Rats in the control and model groups were not treated with drugs. Subcutaneous injection of L-NAME into the back was performed continuously at 125 mg/kg q.o.d. for 5 days from day 14 of pregnancy to induce gestational hypertension. Blood pressure was measured again on day 19 of pregnancy. Finally, rats were anesthetized by intraperitoneal injection of 10% chloral hydrate at 350 mg/kg. No signs of peritonitis were observed. Urine was collected by abdominal compression and *punctio vesicae*. After the fetus was born, rats were sacrificed by cervical dislocation and the placenta was collected. The urine and a portion of the placenta were stored at -80˚C. Another portion of the placenta was fixed in 4% paraformaldehyde at room temperature for 24 h and stored at room temperature.

Experimental grouping. A total of 30 pregnant rats were divided into five groups (n=6): i) Control; ii) model; iii) DMF; iv) succinic acid; and v) DMF + succinic acid. Rats without any treatment served as the control group. Rats in the model group received L-NAME injection but did not receive any drug treatment. Rats that had gestational hypertension and

were treated with DMF were assigned as the DMF group. Rats that had gestational hypertension and were treated with succinic acid were assigned as the succinic acid group. Rats that had gestational hypertension and were treated with both DMF and succinic acid were assigned as the DMF + succinic acid group. In the DMF + succinic acid group, the doses of the two drugs were halved.

Urinary protein determination. Urinary protein was determined using a urinary protein kit, according to the manufacturer's protocol. Briefly, 50 μl water, protein standard solution (563 mg/l) and the urine sample were added into a blank well, standard well and test well, respectively. Coomassie brilliant blue solution (3 ml) was added into each well, followed by mixing. After 5 min, the absorbance (optical density value) was measured at 595 nm using a microplate reader (RT-6100; Rayto Life and Analytical Sciences Co., Ltd.).

Hematoxylin and eosin (H&E) staining. Tissues were fixed in 4% paraformaldehyde at room temperature, washed with running water, dehydrated in 70, 80 and 90% ethanol solution, immersed in an equivalent mixture of absolute ethanol and xylene for 15 min, and transparentized in xylene twice for 15 min each time until transparency. After immersing in an equivalent mixture of xylene and paraffin for 15 min and then in paraffin twice for 50-60 min each time, the tissues were embedded in paraffin and cut into slices, 4-μm thick. Subsequently, the slices were baked, dewaxed, hydrated and then stained with hematoxylin solution for 3 min. Sections were then differentiated in alcoholic hydrochloric acid for 15 sec, washed slightly, blued in bluing buffer for 15 sec, washed with running water, stained in eosin solution at room temperature for 3 min. After staining sections were washed with running water, dehydrated, transparentized, mounted with neutral resin and examined under a light microscope (CKX41; Olympus Corporation).

Immunohistochemical analysis. Tissue slices were prepared as mentioned above and baked at 65˚C for 2 h, immersed in xylene twice for 10 min each, and successively incubated in 100, 100, 95 and 80% ethanol and water for 5 min each time. Subsequently, the slices were incubated in citrate buffer and heated for 2 min in a high-pressure cooker. The slices were then cooled naturally, rinsed with PBS, incubated in fresh 3% hydrogen peroxide at room temperature for 10 min and washed with PBS. After the excess PBS was absorbed by absorbent papers, 5% bovine serum albumin (Beijing Solarbio Science & Technology, Co., Ltd.) was added dropwise onto the slices, which were then incubated at 37˚C for 30 min. After excess blocking buffer was absorbed by absorbent papers, the slices were incubated with primary antibody buffer (rabbit anti-TET1 polyclonal antibody, rabbit anti-KCNMB1 polyclonal antibody) at 4˚C overnight. Following washing with PBS three times, sections were incubated with the secondary antibody buffer (horseradish peroxidase-conjugated goat anti-rabbit IgG) at 37˚C for 30 min. Then sections were rinsed with PBS, developed for 5-10 min using a DAB kit, rinsed with PBS, stained in hematoxylin solution at room temperature for 3 min. Alcoholic hydrochloric acid was used to differentiate and after that sections were blued in bluing buffer, rinsed with water, dehydrated, transparentized, mounted and examined under a light microscope (CKX41; Olympus Corporation).

Table I. The sequences of primers.

Primers	Sequences (5'-3')	Product length (bp)
TET1	F: AAACGGAAGTCAAAACCCC R: CCGAAGAGCCATTGTAAACC	140
KCNMB1	F: AACATCAAGGACCAGGAAGAG R: TTGGTTTTGATCCCGAGTG	129
GAPDH	F: GCAAGTTCAACGGCACAG R: CGCCAGTAGACTCCACGAC	141

TET1, ten-eleven translocation 1; KCNMB1, calcium-activated potassium channel subunit β; F, forward; R, reverse.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Tissue RNA was extracted using TRIzol reagent and RT to cDNA was conducted at 42˚C using the HiFiScript cDNA synthesis kit according to their manufacturer's protocol. Table I presents the primer sequences. The PCR reaction was composed of 1 μl cDNA/DNA, 1 μl forward primer, 1 μl reverse primer, 12.5 μl ULtraSYBR Mixture and 9.5 μl RNase free dH_2O. Reaction parameters included pre-denaturation at 95˚C for 10 min, denaturation at 95˚C for 10 sec, annealing at 58.5˚C for 30 sec, elongation at 72˚C for 30 sec (40 cycles). Analysis parameters of the dissociation curve included 15 sec at 95˚C, 1 min at 58.5˚C, 15 sec at 95˚C, 15 sec at 58.5˚C and 15 sec at 58.5˚C, and measured stepwise from 95˚C, every 0.5˚C. Products were examined using a RT-qPCR detection system (CFX Connect™; Bio-Rad Laboratories, Inc.). GAPDH served as an internal control. Relative levels of genes were calculated using a $2^{-\Delta\Delta Cq}$ method (19).

Western blotting. Tissues were lysed in RIPA lysis buffer at 4˚C for 30 min and then centrifuged at 9,000 x g and 4˚C for 10 min. The supernatant was collected carefully to obtain total protein. Protein concentration was determined using a BCA kit. Subsequently, the protein (24 μg) was denatured and separated by 12% SDS-PAGE for 1-2 h. The protein was transferred to a PVDF membrane by a wet method for 30-50 min, which was then blocked with 3% skimmed milk at room temperature for 1 h and incubated with primary antibody buffer at 4˚C overnight. After washing, the membrane was incubated with secondary antibody buffer at room temperature for 1-2 h, incubated with chemiluminescent substrate and examined on a gel imaging system (ChemiDoc™ XRS+; Bio-Rad Laboratories, Inc.). Gray values were analyzed using Quantity One software (v4.62; Bio-Rad Laboratories, Inc.). GAPDH served as an internal control.

Statistical analysis. Data are presented as the mean ± standard deviation. Every experiment was repeated three times. Data were statistically analyzed using one-way analysis of variance followed by Tukey's post-hoc test with SPSS 19.0 software (IBM, Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

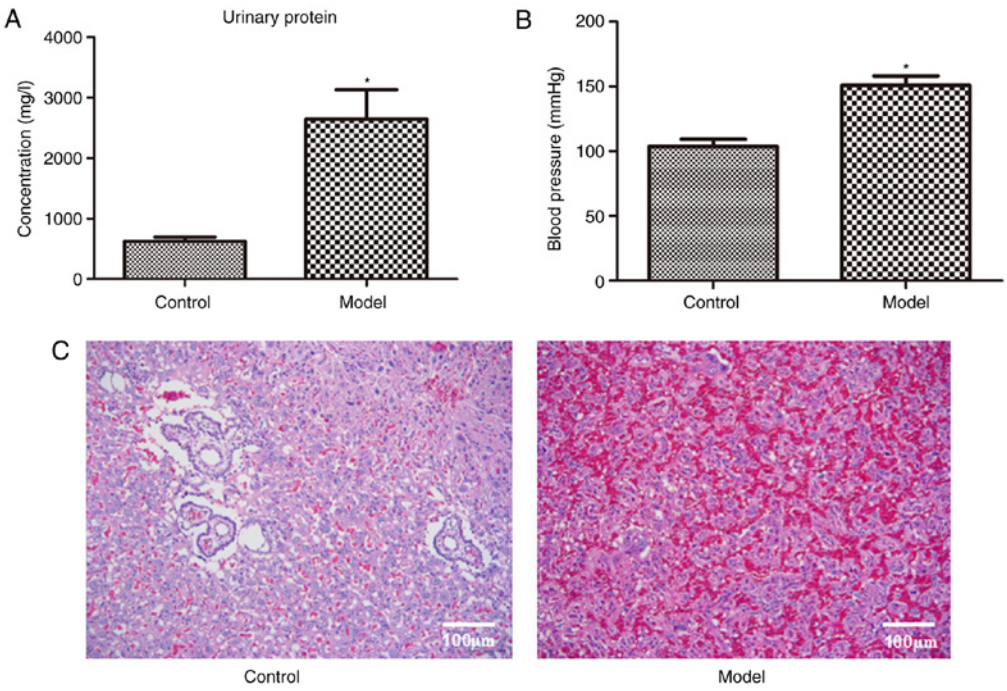

Figure 1. Characterization of gestational hypertension model. (A) Urinary protein, (B) blood pressure and (C) hematoxylin and eosin staining images of the placenta in the control and model rats. *P<0.05 vs. the control.

Results

Establishment of a gestational hypertension model. Fig. 1 presents urinary protein (Fig. 1A), blood pressure (Fig. 1B) and H&E staining images of the placenta (Fig. 1C) in the control and model rats. Compared with the control rats, urinary protein and blood pressure in the model rats increased significantly (P<0.05; Fig. 1A and B). As presented in Fig. 1C, placental cells in the control rats were arranged in an orderly fashion. However, in the model rats, decidual cellular edema of the placenta and vacuolar degeneration were observed, and the intervascular membrane was obviously thicker with a large amount of fibrin deposition. These results indicate that the gestational hypertension model was successfully established.

Urinary protein and blood pressure. Fig. 2 presents the urinary protein (Fig. 2A) and blood pressure (Fig. 2B) of rats in various groups. Compared with the control group, urinary protein and blood pressure in the model group increased significantly (P<0.05). However, compared with the model group, urinary protein and blood pressure in the DMF, succinic acid and DMF + succinic acid groups decreased significantly (P<0.05).

H&E staining. Fig. 3 presents H&E staining images of the placenta in the rats of various groups. Placental cells were arranged in an orderly manner in the control group. However, decidual cellular edema of the placenta, vacuolar degeneration, thicker intervascular membranes and a large amount of fibrin deposition were identified in the model group. Notably, compared with the model group, the edema, vacuoles and fibrin deposition were markedly reduced in the DMF, succinic acid and DMF + succinic acid groups.

Levels of TET1 and KCNMB1. mRNA and protein levels of TET1 and KCNMB1 in the placenta of various groups were examined by immunohistochemical analysis (Fig. 4A), RT-qPCR (Fig. 4B) and western blotting (Fig. 4C). The protein of interest is brown in the immunohistochemical images. Immunohistochemical analysis, RT-qPCR and western blot demonstrated similar results. The levels of TET1 and KCNMB1 were significantly increased in the model groups compared with the control group (P<0.05). However, compared with the model group, their levels were significantly downregulated in the DMF, succinic acid and DMF + succinic acid groups (P<0.05).

Discussion

At present, therapies for gestational hypertension are far from satisfactory. Establishment of an animal model provides a good basis for the investigation of treatments due to the relatively short gestation period in animals. However, the short gestation period and requirement of reserving enough time to treat the disease makes it difficult to establish a model. In the present study, a gestational hypertension model was established by subcutaneous injection of L-NAME. Results of urinary protein and blood pressure measurements demonstrated that urinary protein and blood pressure increased in the model rats. Furthermore, pathological examinations revealed the decidual cellular edema of the placenta, vacuolar degeneration, thicker intervascular membranes and a large amount of fibrin deposition in the model rats. These results confirmed the successful establishment of a gestational hypertension model. L-NAME is an inhibitor of nitric oxide (NO) synthase and can effectively inhibit NO synthesis. NO is an important active substance in maintaining homeostasis in humans and animals. A previous study reported that the NO level in pregnancy is higher than that in the normal state and inhibition of NO synthesis will lead to elevated blood pressure,

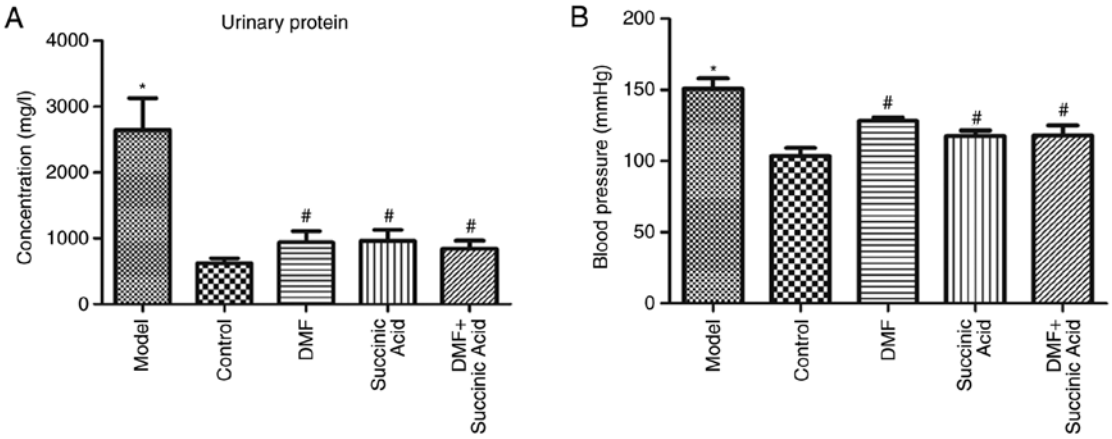

Figure 2. Urinary protein and blood pressure after drug treatment. (A) Urinary protein and (B) blood pressure of rats in the control, model, DMF, succinic acid and DMF + succinic acid groups. *P<0.05 vs. the control; #P<0.05 vs. the model. DMF, dimethyl fumarate.

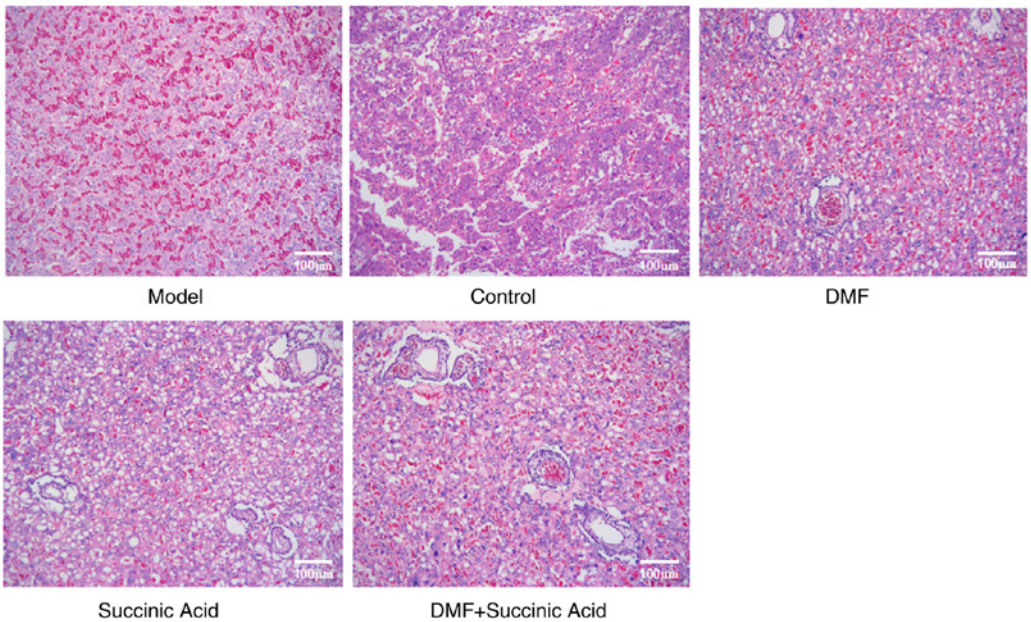

Figure 3. Hematoxylin and eosin staining images of placenta in the rats of the control, model, DMF, succinic acid and DMF + succinic acid groups. DMF, dimethyl fumarate.

proteinuria and symptoms of preeclampsia (20). The results of the present study agreed with this previous study. Elevated blood pressure may be a result of an enhanced response of the vascular system in pregnant rats to angiotensin E and norepinephrine by inhibiting NO synthesis (21).

The present study identified that urinary protein and blood pressure decreased, and placental cells in pregnant rats were improved during gestational hypertension when fumaric acid and succinic acid were administrated. These results suggest that fumaric acid and succinic acid exerted therapeutic effects on gestational hypertension. Non-toxic side effects of fumaric acid and succinic acid in rats are well recognized in previous reports (22,23); therefore, the present study did not perform experiments to confirm their non-toxic side effects in normal non-pregnant female rats.

The TET protein family participates in the whole embryonic development process. When TET1 and TET3 are knocked-out at the same time, the transcriptome diversity increases during early embryonic development (13). Growth defects, increased mortality and developmental retardation are identified in the offspring of paternal mice following TET1-knockout (14). TET1 expression is upregulated during fetal growth, suggesting that TET1 gene may regulate early fetal growth (24). Numerous studies have reported that KCNMB1 reduces the probability of a BKCa channel being open and instantaneous outward potassium current, which weakens the negative feedback inhibitory effect of the BKCa channel and consequently leads to depolarization of the cell membrane, contraction of vascular smooth muscle and elevation of blood pressure (18,25). Correlation analysis between KCNMB1 mutation and hypertension demonstrated that KCNMB1 mutation results in increased sensitivity of the BK channel to calcium ions, which makes blood vessels easier to relax (26). Therefore, the KCNMB1 channel has a negative feedback effect on the contraction of vascular smooth muscle (26). During pregnancy, estrogen upregulates TET1

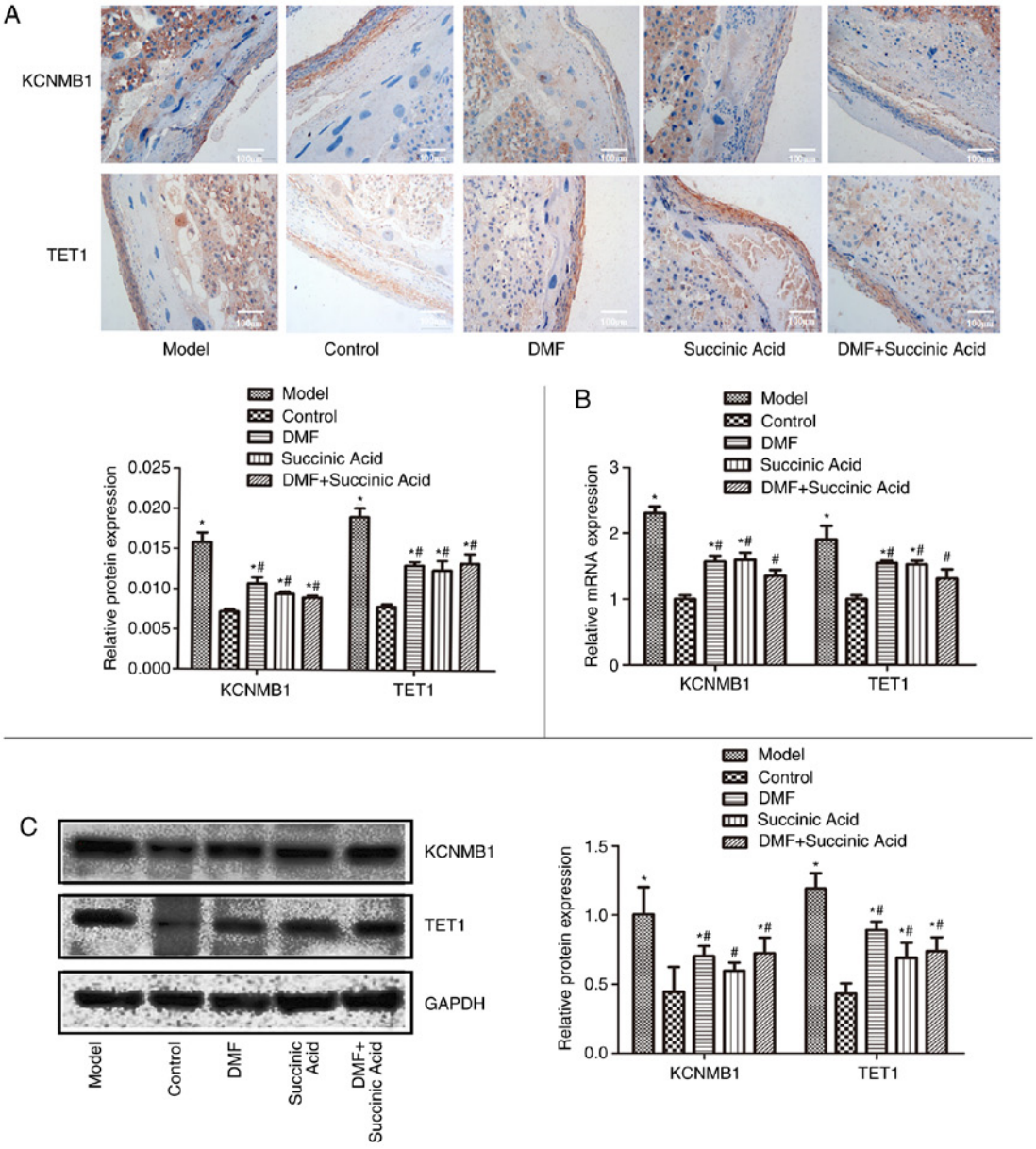

Figure 4. mRNA and protein levels of TET1 and KCNMB1 in the placenta of the control, model, DMF, succinic acid and DMF + succinic acid groups, which were examined by (A) immunohistochemical analysis, (B) reverse transcription-quantitative PCR and (C) western blotting. The protein of interest is brown in the immunohistochemical images. *P<0.05 vs. control; #P<0.05 vs. model. DMF, dimethyl fumarate; TET1, ten-eleven translocation 1; KCNMB1, calcium-activated potassium channel subunit β.

expression in uterine arteries, which activates demethylation and subsequently increases KCNMB1 expression (6). KCNMB1 is associated with hypertension (7). The present results revealed that expression levels of TET1 and KCNMB1 increase during gestational hypertension. This is consistent with the aforementioned reports that TET1 expression is elevated during pregnancy and also suggests that KCNMB1 is associated with hypertension. Fumaric acid and succinic acid are known to inhibit TET (8). In the present study, the expression of TET1 and KCNMB1 decreased following the administration of fumaric acid and succinic acid, suggesting that fumaric acid and succinic acid downregulate the expression of TET1 and KCNMB1.

The absence of histological scoring data was a limitation of the current study. In future studies, promoters or inhibitors will be used to investigate the regulatory relationship between KCNMB1 and TET1 in the treatment of gestational hypertension by fumaric acid and succinic acid, and detect some other preeclampsia-related marker genes/proteins. Additionally, in the present study there were no significant differences for all results between the three treatment groups. This may be due to dosage or other reasons. In the DMF + succinic acid group, the doses of the two drugs were halved, respectively. The reasons underlying the non-significant differences between the three treatment groups is uncertain and requires further experiments to clarify; therefore this will be an aim of future studies.

In conclusion, fumaric acid and succinic acid may treat gestational hypertension by downregulating the expression of KCNMB1 and TET1. Although this requires further confirmation by larger-scale experiments and clinical trials, these results may offer interesting and effective knowledge for the development of drugs to treat gestational hypertension.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and DD designed the study, analyzed the data and wrote the paper. YZ, FZ, HJ, DX and DD performed the study and collected data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Guiyang University of Chinese Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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