

Formic Acid Induces Hypertension-Related Hemorrhage in hSSAO^{TG} in Mice and Stroke Patients

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Abstract

Hypertension is a confirmed risk factor for cerebral hemorrhage in humans. Which endogenous factor directly induces hypertension-related stroke is unclear. In this study, 42 hemorrhagic stroke patients with hypertension and hyperlipidemia and 42 age-matched healthy controls were recruited. The contents of serum semicarbazide-sensitive amine oxidase (SSAO) and formic acid (FC, a final product of SSAO metabolism) were examined in the patients after stroke. Hemorrhagic areas were quantified by computer tomography. In the animal study, hemorrhagic degree was assessed by hemotoxylin & eosin or tissue hemoglobin kits. The relationship between FC and blood pressure/hemorrhagic degree was explored in wild-type mice and hSSAO^{TG} mice fed with high-fat diets or high-fat and -salt diets. The results showed that the levels of serum FC were positively correlated with blood pressure and hemorrhagic areas in stroke patients. Transfection of microRNA-134 could enhance SSAO expression in human vascular smooth muscle cells. Consistently, after treatment with high-fat and -salt diets, hSSAO^{TG} mice exhibited higher levels of miR134 and FC, higher blood pressure, and more severe hemorrhage than wild-type mice. Interestingly, folic acid reduced hypertension and hemorrhage in hSSAO^{TG} mice fed with high-fat diets. These findings suggest that FC is a crucial endogenous factor for hypertension and hemorrhage.

Introduction

The most common causes of intracerebral hemorrhage are hypertension and amyloid angiopathy¹. Epidemiological investigations have revealed that high-fat and -salt diets increase the risk of hemorrhagic stroke². Further studies have shown that high-fat diets can induce hyperlipidemia³, and that high-salt diets directly cause hypertension⁴, and hemorrhagic stroke⁵. Unsurprisingly, hyperlipidemia and hypertension increase the risk of hemorrhagic stroke by several folds⁶. However, the molecular mechanisms of hemorrhagic stroke remain unclear.

The activity and expression of serum semicarbazide-sensitive amine oxidase (SSAO) have been found to be rapidly elevated in ischemia or hemorrhagic stroke patients^{7,8}. MicroRNA-134 (miR134) has been proposed to upregulation induced by high-fat diets⁹, and it can enhance SSAO expression (also named VAP-1) in mice¹⁰. Unexpectedly, overexpression of this gene in the blood vessels causes an abnormal elevation in blood pressure¹¹. Notably, formic acid (FC) is the final product of SSAO metabolism¹²⁻¹⁴. FC administration can induce tissue hemorrhage, blood pressure elevation¹⁵, and dilatation of cerebral vessels¹⁶. These data strongly suggest that SSAO-derived FC may be a direct trigger of hypertension cerebral hemorrhage. Folic acid, a promoting agent of FC metabolism¹⁷, can prevent FC toxicity¹⁸. Thus, it may be used to intervene this disease.

In the present study, we investigated whether high-fat diet increases SSAO expression via miR134/NF- κ B p65 pathway, and test whether endogenous FC (a final product of SSAO metabolism) directly initiates hypertension and hemorrhage in wild-type mice, hSSAO^{TG} mice, and acute stroke patients. Our findings

suggest that SSAO-derived FC endogenously causes hypertension cerebral hemorrhage, and high-salt is a critical exogenous factor for stimulating vascular rupture by enhancing FC toxicity.

Methods

Data collection from human participants.

In this cross-sectional survey, hemorrhagic stroke patients with hypertension and hyperlipidemia (n = 42; age range: 51 ~ 53 years) and age-matched controls (n = 42; age range: 54 ~ 56 years) were recruited from Beijing Boai Hospital in 2018 and 2019 (Chinese Clinical Trial Registry, Clinical Trials: ChiCTR1900026397, China, <http://www.chictr.org.cn/index.aspx>) and. People in northern China preferred high-salt foods. The baseline and clinical characteristics upon admission were recorded, and touch-screen questionnaires were administered, as described in Supplementary Table S1 and Supplementary Table S2 in the Supplement. Informed consent was obtained from each participant either directly or from a guardian before participation. All patients provided written informed consent for study participation. All research was performed in accordance with local policies and guidelines and in accordance with the Declaration of Helsinki. Ethical approval for the clinical investigations was obtained from the Clinical Ethics Committee at the Capital Medical University, China.

Cultured HASMC and HAEC.

HASMC Human aortic smooth muscle cells (*HASMC*) were cultured as described previously³⁰.

HAEC Primary human aortic endothelial cells (*HAEC*, #Clonetics, Allschwil, Switzerland) were cultured and passaged in EBM-2 medium (#EGM-2 bulletkit, Clonetics, Allcell, USA), as described previously³¹.

SSAO expression in HASMC detected by immunofluorescence

HASMC incubated with the treatments for 6 and 12 h were fixed for quantifying SSAO expression³². The primary antibody anti-SSAO (#Sc-166713, 1:200, Santa, USA), goat anti-mouse IgG (H + L) cross-adsorbed secondary antibody, DyLight 633 (#35513, 1:500, Invitrogen, USA), DAPI (#C1002, 1:5000, Beyotime, USA). Slides were viewed under a Zeiss Axioplan microscope (#LSM880, Zeiss, USA).

In vitro cellular toxicity of formic acid and NaCl

The *HASMC* were exposed to the treatments for 6 and 12 h, respectively. The reagents were PBS; sodium chloride (NaCl): 145 mM³³; FC: 10 mM³⁴; and a mixed solution of NaCl and FC. Cell viability was detected at 6 and 12 h with a Cell Counting Kit-8 (#CK04-500T, DOJINDO, Japan). The changes in cellular morphology after FC and NaCl treatment for 6 and 12 h were imaged under a microscope (#IX73, OLYMPUS, USA).

Examination of miR134, NF-kBp65, SSAO by Q-PCR

Total RNA was extracted from samples with TRI Reagent solution (#R1030, Applygen, USA). The quantity of total RNA was determined according to OD260 measurements. *Human miR134 mimic* (sequence (5'-3'): UGUGACUGGUU GACCAGAGGGG, antisense (5'-3'): CCUCUGGUCAACCAGUCACAUU). *U-6 F*: TGCGGTGTGACT GGTTGACCA. *NF-kB p65 F*: TATAGAAGAGCAGCGTGGG GA. *NF-kB p65 R*: GGGGCACGATTGTCAAAGAT. *SSAO F*: AGCAGCAGC GTTTTCAATCAG. *SSAO R*: CCAGGCCACCAAATCCTTTC. *GAPDH F*: GGTCGGAGTCAA CGGATTTG. *GAPDH R*: GGAAGATGGTGTGGGATTTTC. The cDNA was synthesized from total RNA with a Microcute-enhanced microRNA First-chain Synthesis Kit (kr211-02, TIANGEN, USA). Quantitative real-time PCR (qPCR) analysis for microRNA-134 was performed with a Microcute Enhanced MicroRNA Fluorescence Quantitative Detection Kit (#kr411-02, TIANGEN, USA) by Applied Biosystems (#QuantStudio 5, Thermo Fisher Scientific, USA). The expression in the tested samples was normalized to the expression of the housekeeping gene U6³⁵.

AOP-C3, SSAO, H₂O₂, FA, FC, and TG levels tested with kits

The concentrations of Apolipoprotein C-III (AOP-C3, ab154131, Abcam, USA), SSAO (#ab154131, Abcam, USA), H₂O₂ (#S0038, Beyotime, USA), FA (#DFOR-100, BioAssay systems, USA), FC (#K-FORM, Megazyme, Ireland), and triglyceride (TG) (#A110-1-1, Nanjing Jiancheng Bioengineering Institute, China) in serum samples were analyzed with ELISA kits. The optical densities were examined with a microplate reader (#Molecular Devices, SpectramMax i3x, USA).

Hemorrhagic areas of patients analyzed by CT.

The ABC/2 method of computer tomography (CT) allows for rapid estimation of the hemorrhage volume of stroke patients, as described previously³⁶.

Data collection from animal participants.

Generation of hSSAO^{TG} mice

The hSSAO^{TG} mice were made by the Biocytogen Corporation (Beijing, China), as shown in Fig. 4a. The hSSAO^{TG} mice were identified by PCR with DNA primers (F1: 5'-TGTGCTGAGGACTTGTGGTGTAAACC-3'; R1: 5'-CTTGGGAGGCAG CTGCAACTCCA-3') generating a 567-bp product. Ethical approval for these animal studies was obtained from the Ethics Committee at the Capital Medical University, China.

Body mass index of mice

To generate the growth curve, the weights of the mice were measured once per week. Body weight and length (from the nose to anus) were measured at 8 weeks. Body mass index (BMI) was calculated as body weight (g)/body length² (cm)³⁷.

Hemorrhagic areas of mice stained by H&E

Frozen brain samples were sectioned at 15 μm thickness and stained with hematoxylin & eosin (H&E)³⁸.

Quantification of brain vascular rupture by detecting brain hemoglobin

Brain tissues were homogenized in Drabkin's color reagent according to the manufacturer's instructions (#D5941, Sigma-Aldrich, USA). The extravagated blood in the tissue homogenates was quantified at 540 nm with a spectrophotometer (#Multi-Mode Microplate Reader, SpectraMax i3, Molecular Devices, USA).

Hemorrhagic stroke of mice examined by MRI

MRI measurements were made with a PharmaScan 7T small-animal MR scanner and a four-channel mouse brain surface coil (#Bruker Biospin, Ettlingen, Germany) as described previously³⁹.

SSAO expression in blood vessels stained by immunofluorescence

The immunofluorescence was carried out as previous report⁴⁰. The primary mouse polyclonal antibody against VAP-1/SSAO (#Sc-166713; 1:200, Santa Cruz, USA), goat anti-mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, DyLight 633 (#35513, 1:500, Invitrogen, USA).

Establishment of acute and chronic animal models

Acute models Three groups of adult male wild-type C57/BL-6J mice (n = 12, per group) were intraperitoneally injected with the treatments (saline; high-salt: 8% NaCl daily⁵; FC: 5‰, 0.2 mL/10 g¹⁴; and mixed solution of NaCl and FC) for 2 weeks.

Chronic models The hSSAO^{TG} (n = 10) and wild-type mice (n = 10) were fed high -fat and/ -salt diets or folic acid (oral gavage, 1mg/kg/day weeks) for 8 consecutive weeks, as described previously⁴¹.

Blood pressure measurement

Blood pressure monitors (#BP-2010 Series, Softron, Japan) were used to measure systolic blood pressure.

Statistical Analyses.

All data were tested for normality with the Kolmogorov-Smirnov test. When data were normally distributed, the statistical significance of differences was assessed with the unpaired *t* test and one, *one-way* or *two-way* ANOVA, analyzed by Sidak's multiple comparisons test. When the data were not normally distributed, the statistical significance of differences was assessed on the basis of *P* values with the Mann-Whitney *U* test. The human serum biochemical index was assessed with Student's unpaired *t*-test. The sex of participants was assessed with the chi-squared test. Human blood pressures were presented as the median, and *p*-values were assessed with the Mann-Whitney *U*-test. Correlations between the levels of serum SSAO/formic acid and hemorrhagic degree were assessed with the Pearson correlation coefficient, both without adjustment and with adjustment for sex and age. Statistical significance was set

to $p < 0.05$. Analyses were performed in GraphPad Prism 6 software (GraphPad PRISM software, version 6.01, USA).

Result

Positive correlation of formic acid levels and blood pressure/hemorrhagic areas in stroke patients.

Previous studies have shown that SSAO is present in the blood and vascular smooth muscle cells¹⁹, which can catabolize methylamine into ammonia, formaldehyde (FA, HCHO), and hydrogen peroxide (H_2O_2)^{12,13}; then the final product, formic acid (FC), is formed after FA oxidation (Fig. 1a, b)¹⁴. We quantified the concentrations of serum SSAO, FA, H_2O_2 , and FC, and found that serum FA and H_2O_2 levels were lower in 42 stroke patients than 42 age-matched controls (Fig. 1c, d); while FC levels in stroke patients were higher than that of controls (Fig. 1e).

To confirm that these patients suffered from hemorrhagic stroke, low levels of serum Apolipoprotein C-III (AOP-C3) as a marker of hemorrhagic stroke²⁰, were detected. As expected, AOP-C3 levels in 42 stroke patients were lower than controls (Supplementary Fig. S1a), and the serum SSAO levels were negatively correlated with the AOP-C3 levels in 84 participants (Supplementary Fig. S1b). Furthermore, there was a positive correlation between serum FC levels and mean blood pressure (MBP, $MBP = (DBP + SBP) / 2$) (Fig. 1f and Supplementary Fig. S1c, d). Notably, abnormal systolic blood pressure ($SBP > 140$ mmHg) and diastolic blood pressure ($DBP > 90$ mmHg) were observed in these stroke patients (Supplementary Fig. S2).

Stroke cases were further conformed by computed tomography (CT) and hemorrhagic areas analyzed by the ABC/2 method (Fig. 1g, top). There was a positive correlation between hemorrhagic areas and the levels of serum SSAO, H_2O_2 , and FC, respectively (Fig. 1g-i). Hence, SSAO-derived FC is closely related with hypertension and hemorrhage (Fig. 1j).

Formic acid-induced cell swelling, hypertension and hemorrhage in wild-type mice. To determine which factor is the possible trigger of cerebral hemorrhage, we examined the changes of the up- and down-stream molecules of SSAO in stroke patients. High-fat diets upregulates microRNA-134⁹, and enhances SSAO expression (Fig. 2a)¹¹. Hence, we examined these molecules, such as miR134 by Q-PCR, SSAO tested by human SSAO kits, and FC detected by FC kits. We found that the levels of miR134, SSAO, and FC in the patients after stroke onset 2 ~ 3 hours were higher than that after 4 ~ 6 months (Fig. 2a-d).

Excessive FC could directly elicit blood pressure elevation¹⁵, suggesting that FC may be a candidate trigger. The results showed that FC incubation led to cell swelling of human aortic smooth muscle cells (*HASMC*) at 6 and 12 hours (Fig. 2e and Supplementary Fig. 3a); while high salt (NaCl, a positive control, a trigger of hypertension and hemorrhage) with FC synergistically exacerbated cell swelling and death compared with cells receiving only NaCl or FC treatment (Fig. 2f). Similarly, high salt with FC synergistically also enhanced cell swelling and death in the cultured human vascular endothelial cells

(HVEC) (Supplementary Fig. S3b, c). In addition, NaCl accelerated FC release from *HASMC* when the culture medium contained FC at 6 h (Fig. 2g).

To address whether FC directly induced hypertension *in vivo*, FC was intraperitoneally injected into wild-type mice for consecutive two weeks, blood pressure was monitored by electric blood pressure meter (Supplementary Fig. S4), hemorrhagic areas were quantified by H&E, and brain vascular rupture was examined by tissue hemoglobin kits. Unexpectedly, an elevation in systolic blood pressure (SBP) was observed in these mice injected with FC, NaCl, or FC plus NaCl; while high salt accelerates FC-induced hypertension (Fig. 2h and Supplementary Fig. S5).

Of note, FC as well as high salt (NaCl) directly elicited hemorrhagic stroke; while FC combined with NaCl resulted in greater hemorrhagic areas than did only FC or NaCl (Fig. 2i). Hemoglobin as a marker of cerebral hemorrhage is based on that hemoglobin flows out from the ruptured blood vessels²¹. In this study, FC directly caused a marked elevation in brain hemoglobin levels (Fig. 2j), indicating brain vascular rupture was occurred.

Upregulation of SSAO by high-fat diet-derived miR134 in the cultured *HASMC*, wild-type mice, and hSSAO^{TG} mice. To address whether SSAO overexpression is mediated by miR134/NF- κ B p65 pathway (Fig. 3a)^{22,23}, we transfected mimics of miR134 into cultured *HASMC*. The changes in mRNA levels of NF- κ B p65 and SSAO were examined by Q-PCR; their protein contents were quantified by immunofluorescence (I.F.) (Fig. 3b). As expected, mimics of miR134 improved mRNA levels of both NF- κ B p65 and SSAO (Fig. 3c, d), and increased the fluorescence intensity of NF- κ B p65 and SSAO than controls (Fig. 3e-g and Supplementary Fig. S6). Thus, miR134 can enhance SSAO expression *in vitro*.

To test whether miR134 and SSAO contributes the occurrence of hemorrhagic stroke *in vivo*, we generated transgenic mice with overexpressing human *SSAO* gene (hSSAO^{TG}) (Fig. 4a, and Supplementary Fig. S7-9). In fact, high-fat diets or high-fat and -salt diets markedly increased body weights body mass index (BMI), and serum triglyceride (TG) in hSSAO^{TG} mice than wild-type mice (Fig. 4b, c). Notably, high-fat diets obviously increased miR134 levels in wild-type mice; however, high-fat and -salt diets induced higher levels of miR134 in hSSAO^{TG} mice than wild-type mice (Fig. 4d).

Through immunofluorescence with anti-SSAO, we found that high-fat diet or high-fat and -salt diets increased SSAO expression in the vascular walls and vascular abnormalities in thoracic aorta and cerebral microvessels (Fig. 4e, f, and Supplementary Fig. S10). Taken together, high-fat diet enhances miR134-mediated SSAO expression (Supplementary Fig. S11).

Formic acid- a direct trigger of hypertension/hemorrhage in hSSAO^{TG} Mice and wild-type mice. To find out the roles of endogenous FC in hypertension cerebral hemorrhage in wild-type mice and hSSAO^{TG} mice fed with high-fat diet or high-fat and -salt diets for 8-weeks, we investigated the relationship between FC levels and blood pressure/hemorrhage.

The results showed that the levels of serum H_2O_2 and FA were declined (Fig. 5a, b); while serum FC levels were elevated in these two kinds of mice fed with different diets than those mice treated with the standard diet (Fig. 5c). Furthermore, we found that high-fat and -salt diets induced higher SBP and lower DBP in $hSSAO^{TG}$ mice than wild-type mice (Fig. 5d-f). Especially, serum FC levels were positively correlated with SBP in these six groups' mice (Fig. 6a).

Remarkably, the results of H&E staining revealed that high-fat and -salt diets caused greater hemorrhagic areas in $hSSAO^{TG}$ mice than wild-type mice (Fig. 6b, c). Meanwhile, more severe brain vascular rupture was occurred in the former than the latter, because the former had higher levels of brain hemoglobin (Fig. 6d). Similarly, this conclusion was confirmed by magnetic resonance imaging (MRI), in which two black cycle shadows were observed in the brains of $hSSAO^{TG}$ mice fed with high-fat and -salt diets compared with wild-type mice (Fig. 6e).

Further statistical analysis showed that serum FC levels were positively correlated with hemorrhagic degree detected by H&E or tissue hemoglobin kits, respectively (Fig. 6f). Notably, the higher levels of FC lead to the earlier onset of stroke in $hSSAO^{TG}$ mice than the wild-type mice fed with the same high-fat and -salt diets (Stroke onset: 3 weeks vs. 8 weeks) (Fig. 6g). Based on the above data, we concluded that endogenous FC is a crucial trigger of hypertension and hemorrhage.

Rescue effects of folic acid on hypertension/hemorrhage in acute or chronic formic acid-related model mice. If FC is a direct trigger of hypertension and hemorrhage, reduction of FC or enhancement of its metabolism should alleviate hemorrhagic stroke. Folic acid was selected to reduce FC-induced vascular toxicity, because it is a promoting agent of FC metabolism, which transfers FC to CO_2 and H_2O by activating the enzyme of 10-formyl tetrahydrofolate synthetase (THF)¹⁷.

As expected, 2-week injection of folic acid decreased SBP in FC- or NaCl-injected wild-type mice (Fig. 7a, b and Supplementary Fig. S12). Hemorrhagic areas were also reduced in those mice after folic acid treatment (Fig. 7c and Supplementary Fig. S13). Similarly, oral gavage of folic acid for 8 weeks could restore SBP (Fig. 7d and Supplementary Fig. S14), reduced hemorrhagic areas (Fig. 7e), and decreased brain vascular rupture in $hSSAO^{TG}$ mice fed with high-fat diets (Fig. 7f).

Discussion

The first important result obtained in this study is that SSAO-derived FC endogenously induces hypertension and hemorrhage, and exogenous high-salt intake accelerates FC-induced vascular rupture. However, reduction of FC by folic acid could alleviate hypertension cerebral hemorrhage (Fig. 8).

Blood vessel breakage in the brain is the direct reason for hemorrhagic stroke²⁴. High-fat diets have been found to change the profile of microRNAs including miR134⁹, and to enhance SSAO expression in blood vessels. MiR134 activates NF- κ B²², and the phosphorylation of NF- κ B p65 upregulates SSAO. In the

present study, we provided evidence that miR134 upregulated SSAO in the cultured *HASMC*, wild-type mice and hSSAO^{TG} mice fed with high-fat diet, especially, in hemorrhagic stroke patients.

Another interesting finding was that serum FC (> 0.47 µg/mL) caused hypertension and hemorrhage in both mice and humans. Endogenous FC is mainly generated from the metabolism of SSAO-derived FA and/or methanol. Unsurprisingly, clinical cases show that exogenous methanol can induce hypertension^{25,26}, and hemorrhage^{27,28}. Actually, acute or chronic FC increased SBP in wild-type mice and hSSAO^{TG} mice. Especially, higher levels of FC were associated with earlier stroke onset; and FC still remained relatively high levels after stroke. Hence, scavenging FC may contribute to stroke recovery.

A potential limitation of this study pertains to the cross-sectional design. The blood samples from these inpatients before stroke did not obtain, because there's no way to predict who will suffer from stroke. Thus, longitudinal studies in community population will contribute to the establishment of the relationship between FC levels and blood pressure/hemorrhagic degree.

Folic acid could enhance FC metabolism and prevent hemorrhagic stroke in mice. Daily supplementation with folic acid may effectively prevent stroke in humans. High-salt accelerates FC-induced stroke. Lower-salt diets decrease the risk of ischemia and hemorrhagic stroke²⁹. Hence, FC may be a promising therapeutic target for preventing and/or treating hypertension-related hemorrhage.

Declarations

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Authors' contributions Dr Yan Yu and Zhiqian Tong had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Yalan Di and Yan Yu contributed equally to this work as co-first authors. Rongqiao He, Jianjun Li, Zhiqian Tong are the co-corresponding authors in this study.

Competing interests

The authors declare no competing interests.

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Figures

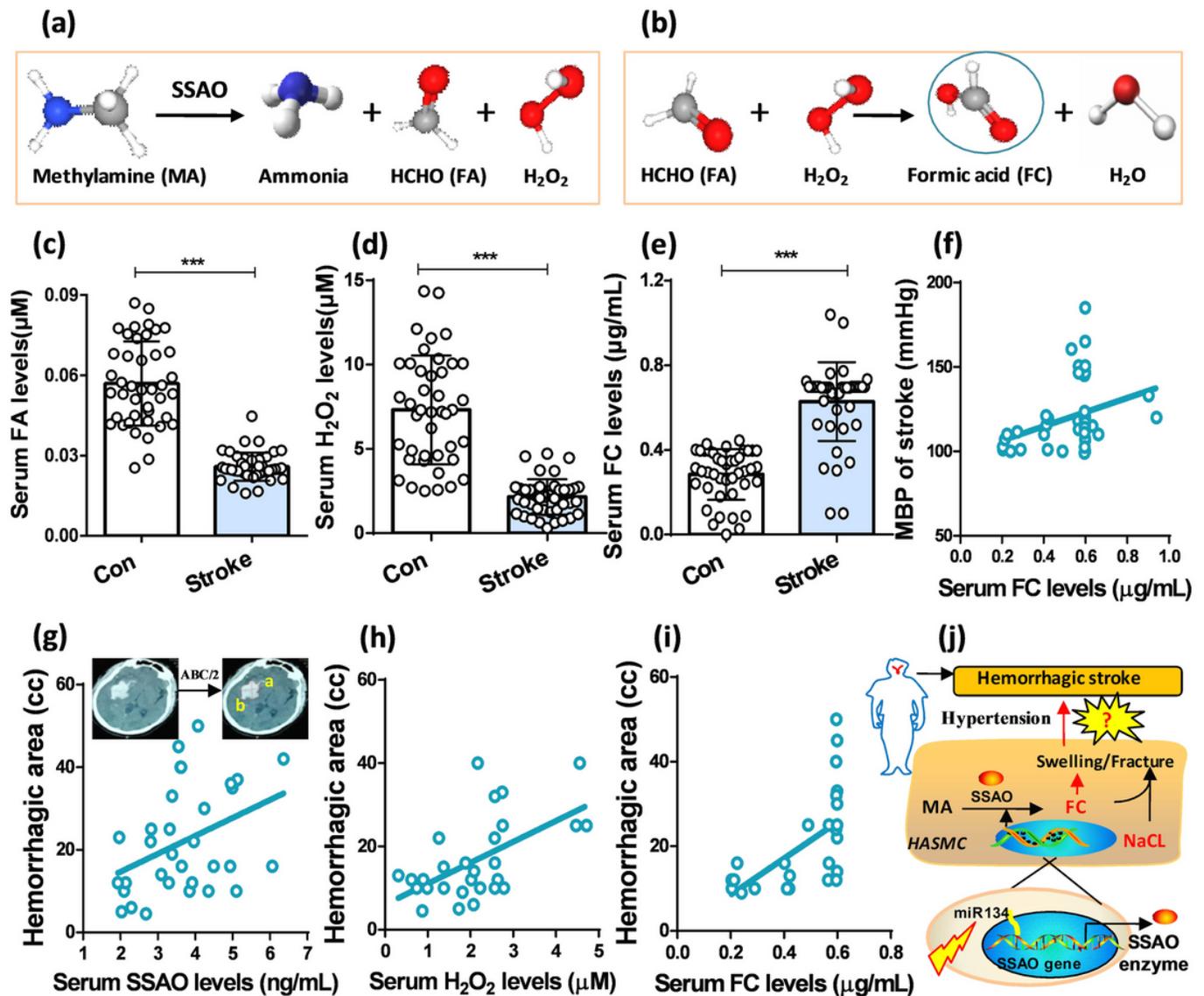


Figure 1

Serum formic acid levels are positively correlation with blood pressure and hemorrhagic areas in stroke patients. (a) SSAO catabolizes methylamine to generate ammonia, formaldehyde (FA) and H₂O₂. (b) FA is oxidized to formic acid (FC) by H₂O₂. (c), (d) Decline in the levels of serum FA and H₂O₂ in stroke patients. (e) Elevation in the levels of serum FC. *** p < 0.001, by unpaired, 2-tailed Student's t test. (f) Positive correlation between the levels of serum FC and MBP, n=42. $y = 41.97x + 98.07$; $F = 5.414$, $R^2 = 0.119$; $p = 0.0251$. *p < 0.05, correlation coefficient and equation analyzed by linear regression test. (g) The hemorrhagic areas quantified by using ABC/2 method to analyze CT (top), and negative correlation between hemorrhagic areas and serum SSAO levels in 84 participants (bottom). ($y = 4.349x + 6.004$, $R^2 = 0.155$, $F = 5.497$, $p = 0.0259$). (h), (i) Negative correlation between the levels of

serum H2O2/FC and hemorrhagic areas. H2O2 ($y = 5.018x + 6.062$, R square=0.324, F=12.96, p=0.0013); FC ($y = 42.98x - 0.0614$, R square = 0.332, F=12.95, p=0.0013). FC: formic acid. n=42. (j) The assumed model of SSAO- induced hemorrhagic stroke. SSAO: semicarbazide-sensitive amine oxidase; MA: methylamine; FC: formic acid; miR134: microRNA-134. Data in the dot plots represent the mean \pm SD.

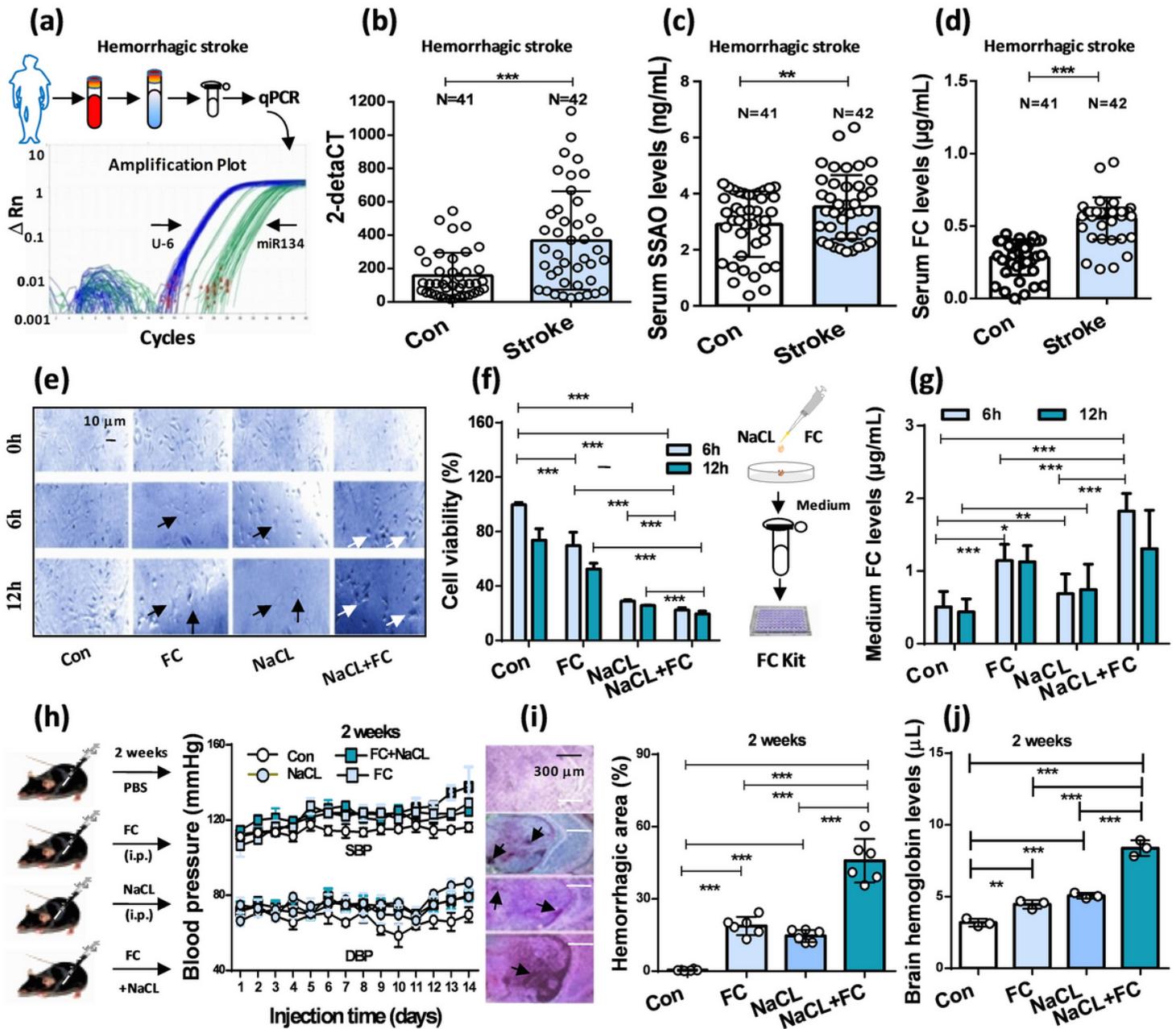


Figure 2

Excessive formic acid induces cell swelling of vascular smooth muscle and hypertension cerebral hemorrhage in the wild-type mice. (a) The experimental process of detecting serum miR134 in stroke patients. (b)-(d) miR134 quantified by Q-PCR (b), SSAO levels detected by human SSAO kit (c) serum FC levels examined by FC kit (d) in 42 patients and 42 age-matched controls. ***p<0.0001, by two-way ANOVA with Sidak's multiple comparisons test. (e) HASMC swelling induced by FC, NaCL, and FC

combined with NaCL at 0, 6, and 12 h, respectively (bar=10 μ m). HASMC: human aortic smooth muscle cells. (f), (g) NaCL and FC synergistically enhanced HASMC death and intracellular FC release at 6 and 12 h; n=6. **p<0.001, ***p<0.001, by unpaired, 2-tailed Student's t test. (h) The changes in blood pressure in the four groups' mice after 2-week injection with PBS, FC, NaCL, and FC plus NaCL. FC: formic acid. PBS: phosphate buffered saline. SBP: systolic blood pressure; DBP: diastolic blood pressure; n=6. (Group factor: F(7, 546)=696.7, p<0.0001; time factor: F(13,546)=5.305, p<0.0001, interaction: F(91, 546)=1.249, p=0.0720). *p<0.05, ***p<0.0001, data analyzed by two-way ANOVA with Sidak's multiple comparisons test. (i) Hemorrhagic areas quantified by H&E. n=6. (j) Brain vascular rupture detected by tissue hemoglobin kits. n=3. **p<0.01, ***p<0.001, data analyzed by repeated-measures two-way ANOVA with Bonferroni's correction. Data in the dot plots represent the mean \pm SD.

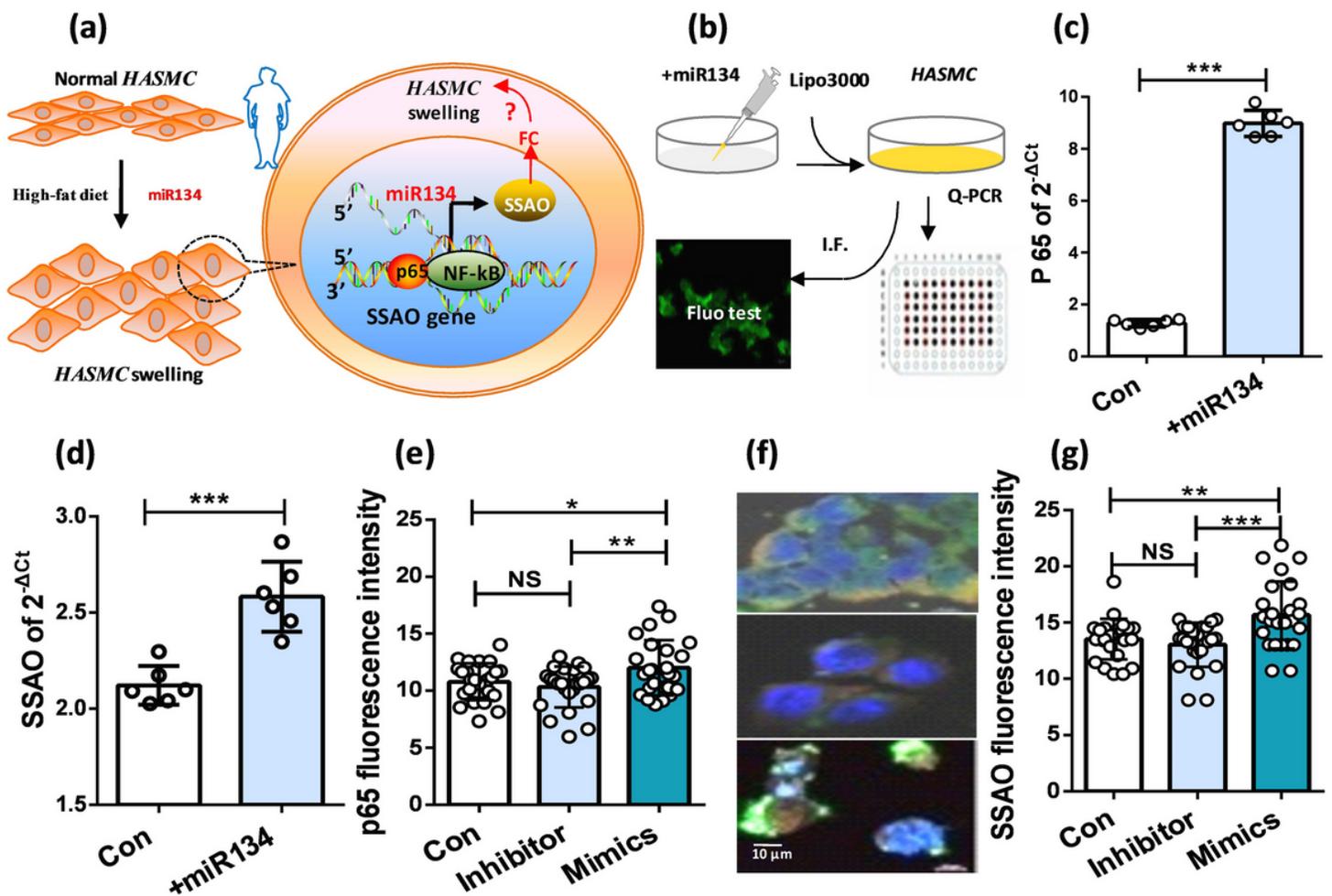


Figure 3

High-fat diet-derived microRNA-134 enhances SSAO expression in the cultured HASMC. (a) The assumed model of high-fat diet-induced miR134 upregulating SSAO. miR134: microRNA-134; HASMC: human aortic smooth muscle cells; SSAO: semicarbazide-sensitive amine oxidase; FC: formic acid. (b) The experimental process of transfection of miR134-induced SSAO overexpression in the cultured HASMC. HASMC: human aortic smooth muscle cells; SSAO: semicarbazide-sensitive amine oxidase; miR134: microRNA-134. I.F.: immunofluorescence. (c), (d) Quantification of mRNA levels of NF-κB p65 and SSAO

by Q-PCR. n=6. (e)-(g) Statistical analysis of the fluorescence intensity of NF- κ B p65 and SSAO. SSAO (red), NF- κ B p65 (yellow), GFP as a green reporter gene in the control plasmid, DAPI as a nuclear dye. n=26, ***p<0.0001, by unpaired two-tailed Student's t test.

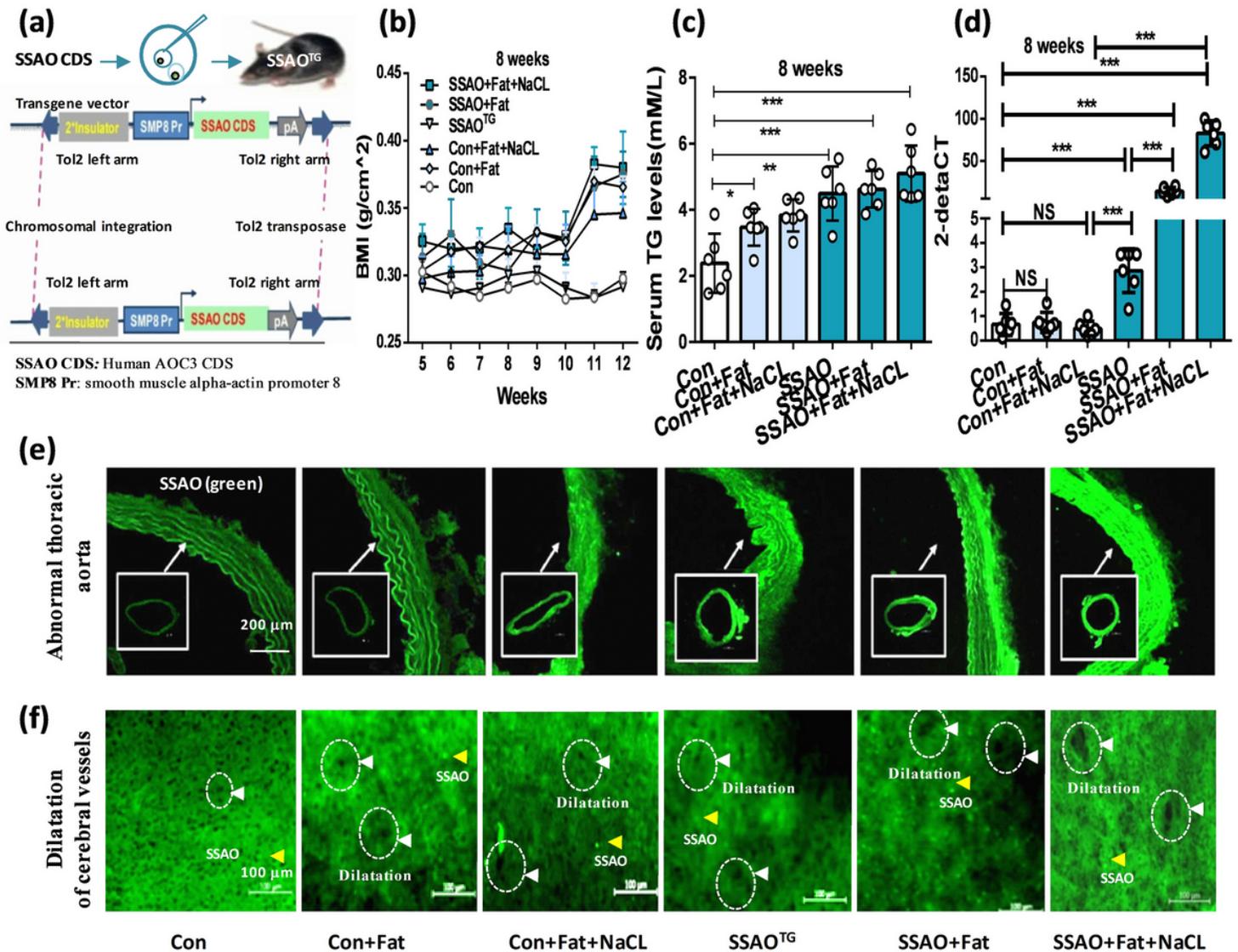


Figure 4

High-fat diet- derived miR134 upregulates SSAO in wild-type mice and hSSAOTG mice. (a) The transgenic technology roadmap for generating hSSAOTG mice. SMP8 Pr: smooth muscle alpha-actin promoter 8. (b) The changes in BMI in these six groups' mice. n=6, ***p<0.0001, data analyzed by two-way ANOVA with Sidak's multiple comparisons test. BMI: body mass index; Con: wild-type mice; Con+Fat: wild-type mice fed high-fat diets; Con+Fat+NaCL: wild-type mice fed with high-fat and -salt diets; SSAO: mice transfected with human SSAO gene (hSSAOTG); SSAO+Fat: hSSAOTG mice fed with high-fat diets; SSAO+Fat+NaCL: hSSAOTG mice fed with high-fat and -salt diets. (c), (d) The changes in the levels of serum TG and miR134. n = 6. **p<0.01, ***p<0.001, Data analyzed by repeated-measures two-way ANOVA with Bonferroni's correction. (e), (f) The changes in SSAO expression (green) and vascular structures of the thoracic aorta (e) (bar=200 μ m) and cerebral microvessels (f) (bar=300 μ m) in these six groups' mice.

Microvessel dilatation was marked with a white triangle. These experiments were repeated three times. Data in the dot plots represent the mean \pm SD.

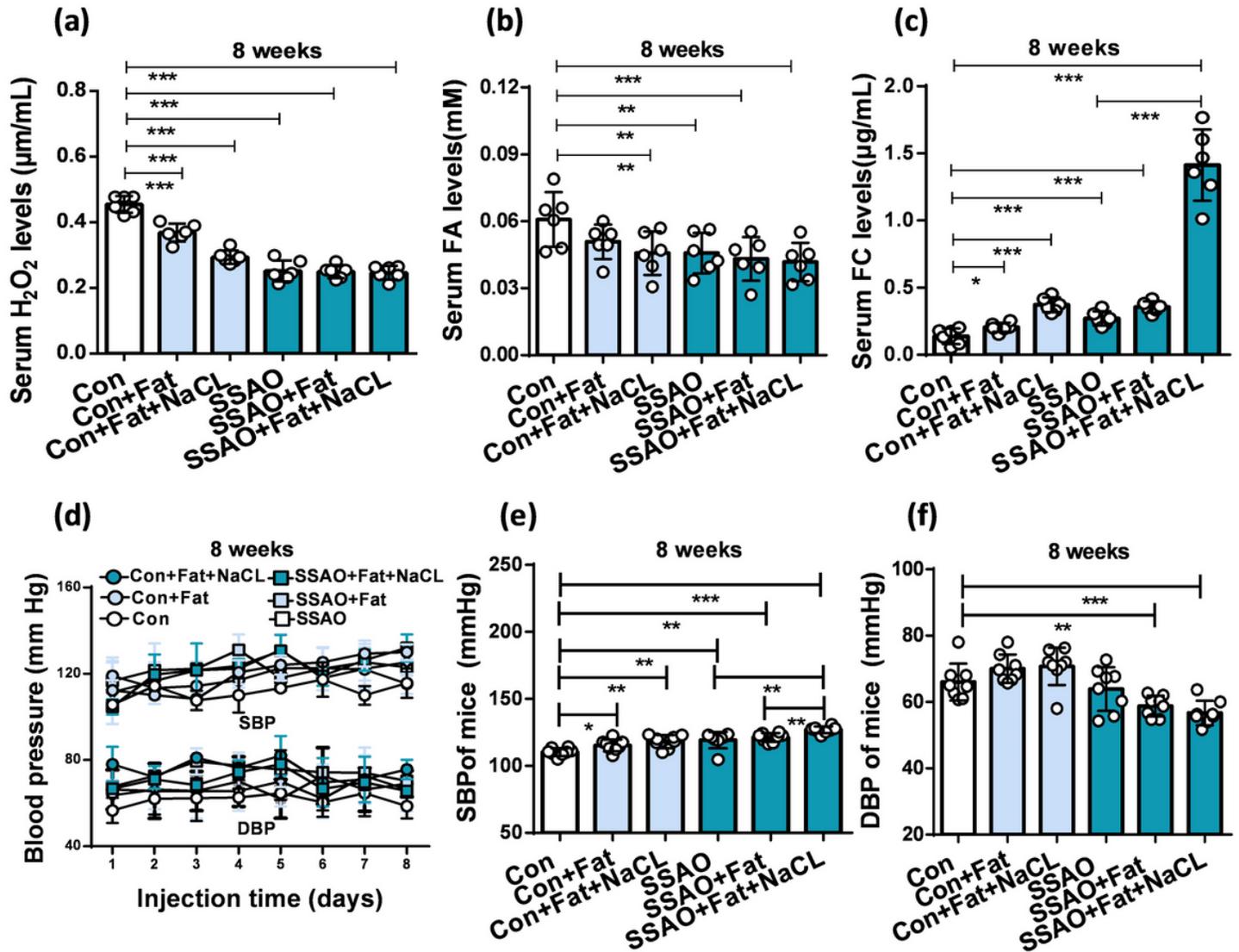


Figure 5

Higher levels of serum formic acid are associated with severe hypertension in wild-type mice and hSSAOTG mice fed with special diets. (a)-(c) Detection of the concentrations of serum H₂O₂ (a), FA (b) and FC (c) in these six groups' mice. Con: wild-type mice; Con+Fat: wild-type mice fed high-fat diets; Con+Fat+NaCL: wild-type mice fed with high-fat and -salt diets; SSAO: mice transfected with human SSAO gene (hSSAOTG); SSAO+Fat: hSSAOTG mice fed with high-fat diets; SSAO+Fat+NaCL: hSSAOTG mice fed with high-fat and -salt diets. n=6. *p<0.05, **p<0.001, ***P<0.001, by one-way ANOVA with Bonferroni's correction. (d) Measurement of blood pressure by electric blood pressure meter. SBP: systolic blood pressure; DBP: diastolic blood pressure. n=6, (group factor: F(7,192)=6.430, p<0.0001; time factor: F(11,192)=334.8, p<0.0001, interaction: F(77,192)=1.668, p=0.0026. ***p<0.0001, data analyzed by two-way ANOVA with Sidak's multiple comparisons test. (e), (f) Changes in SBP and DBP in these six groups' mice. n=6, **p<0.001, ***P<0.001 by one-way ANOVA with Bonferroni's correction.

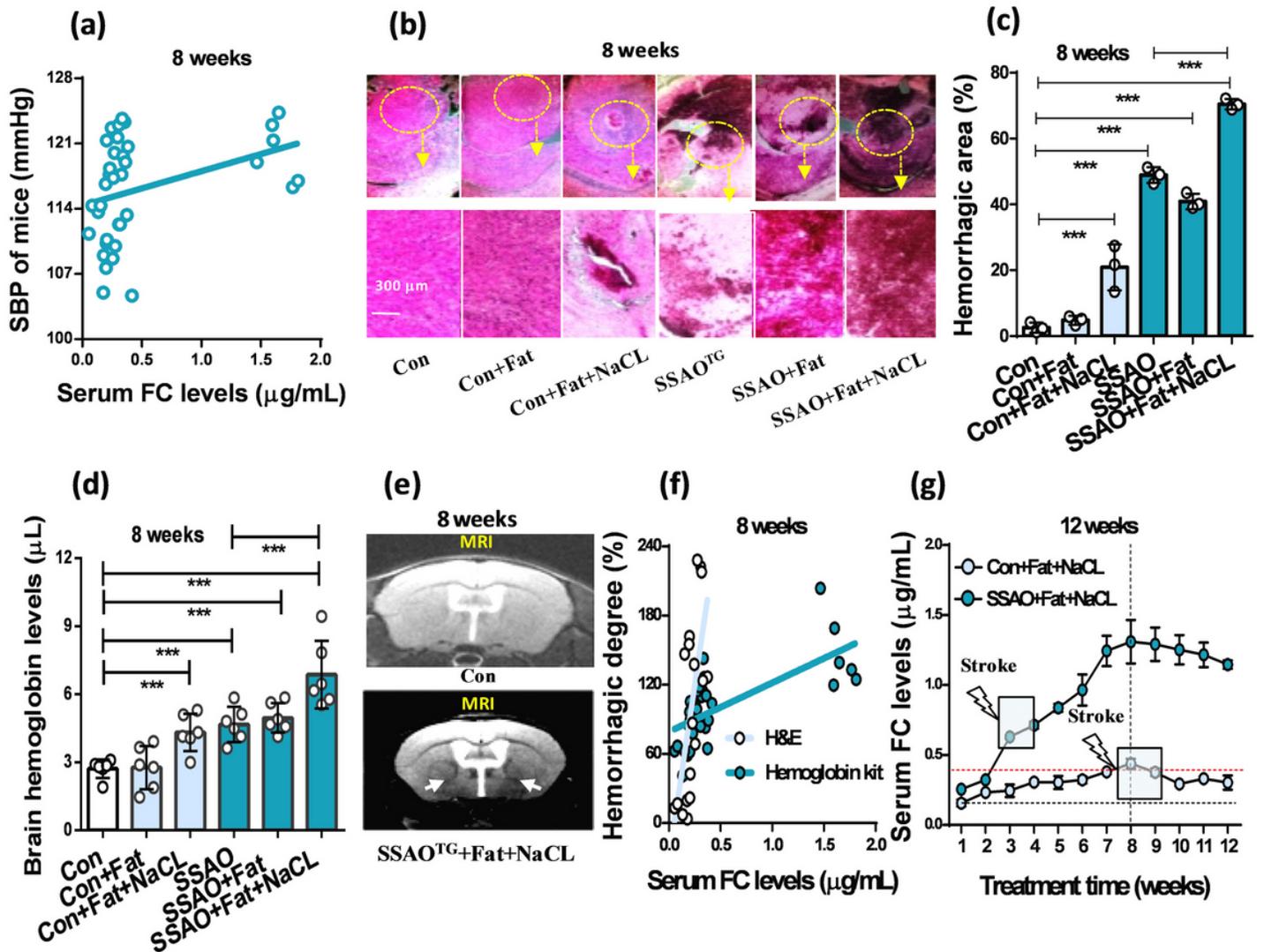


Figure 6

Higher levels of serum formic acid are accompanied by severe hemorrhage in wild-type mice and hSSAOTG mice fed with special diets. (a) Positive correlation between FC levels and SBP. $n=6$, $y=3.683x+114.4$, $R\text{ square}=0.1247$, $p=0.0338$, $*p<0.05$, correlation coefficient and equation analyzed by linear regression test. (b), (c) Hemorrhagic areas stained by hematoxylin and eosin (H&E) (bar=300 μm). $n=3$. (d) Brain vascular rupture examined by tissue hemoglobin kit. $n=6$. $***P<0.001$, by one-way ANOVA with Bonferroni's correction. (e) Identification of hemorrhage by magnetic resonance imaging. MRI: magnetic resonance imaging. (f) Positive correlation between serum FC levels and hemorrhagic degree quantified by these two methods. H&E ($y=606.9x-31.76$, $R\text{ square}=0.4190$, $p=0.007$); Hemoglobin ($y=42.39x+79.32$, $R\text{ square}=0.4761$, $p<0.0001$), $**p<0.01$, $***p<0.0001$, correlation coefficient and equation analyzed by linear regression test. (g) Changes in serum FC levels in hSSAOTG mice and wild-type mice fed with high-fat and -salt diets for consecutive 12 weeks. $n=3$, $***p<0.001$, data analyzed by two-way ANOVA with Sidak's multiple comparisons test. Data in the dot plots represent the mean \pm SD.

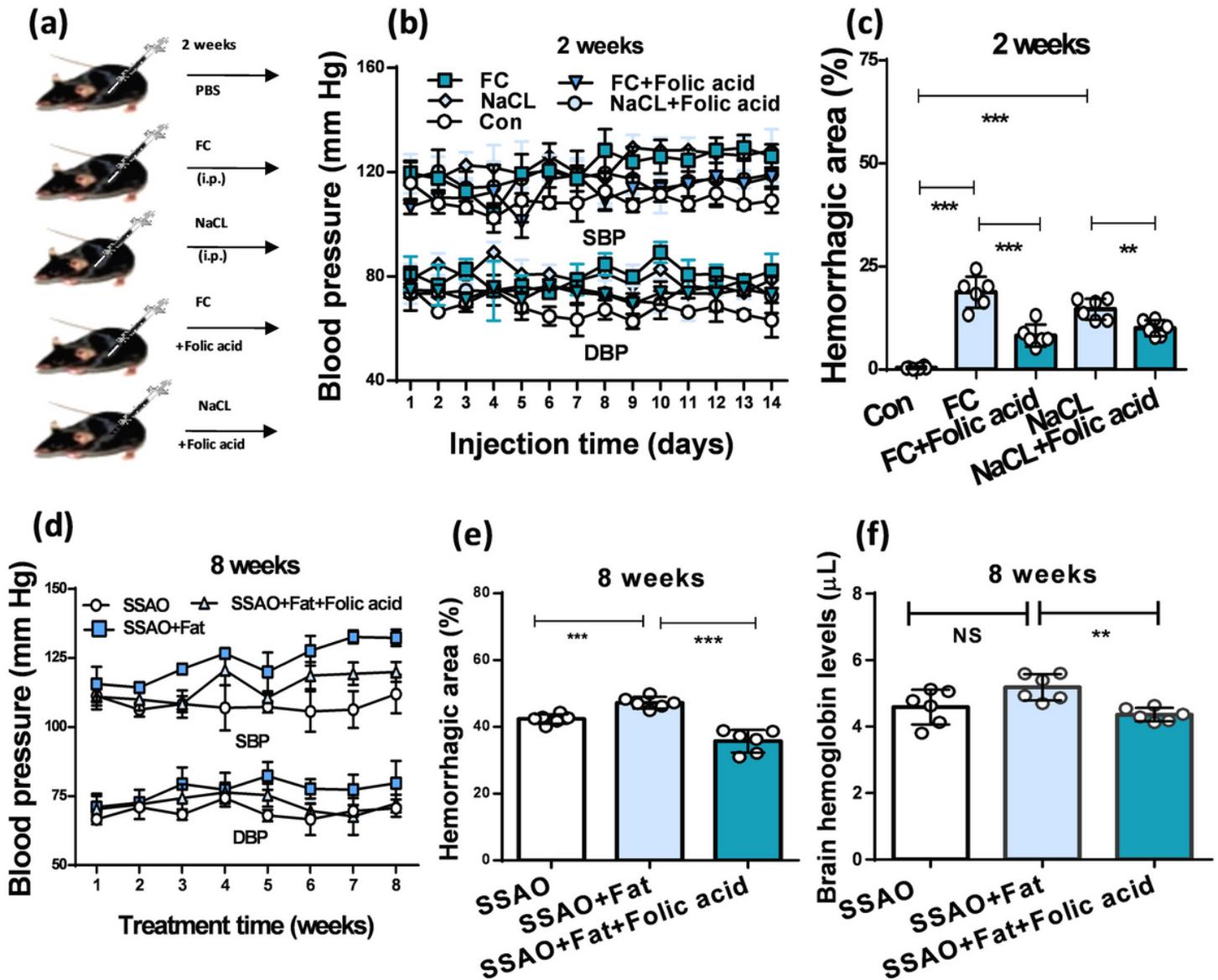


Figure 7

Folic acid alleviates formic acid- induced hypertension and hemorrhage in acute or chronic model mice. (a), (b) The changes in blood pressure in mice after 2-week injection with PBS, FC, NaCL, FC plus folic acid, and NaCL plus folic acid. FC: formic acid. PBS: phosphate buffered saline; n=6. Group factor: $F(9,280)=665.8$, $p<0.0001$; Time factor: $F(13, 280)=1.509$, $p=0.1133$; interaction: $F(117,280)=1.569$, $p=0.0014$. ** $p<0.001$, *** $p<0.0001$, data analyzed by two-way ANOVA with Sidak's multiple comparisons test. SBP: systolic blood pressure; DBP: diastolic blood pressure. (c) Hemorrhagic areas stained by hematoxylin and eosin (H&E). n=6. *** $P<0.001$, by one-way ANOVA with Bonferroni's correction. (d) The changes in blood pressure in hSSAOTG mice with or without high-fat diet or folic acid treatment. n=6. Group factor: $F(5,96)=480.6$, $p<0.0001$; Time factor: $F(7,96)=3.942$, $p=0.0008$; interaction: $F(35, 96)=1.569$, $p=0.0447$. * $p<0.05$, *** $p<0.0001$, data analyzed by two-way ANOVA with Sidak's multiple comparisons test. (e) Hemorrhagic areas stained by H&E. n=6. *** $P<0.001$, by one-way ANOVA with

Bonferroni's correction. (f) Brain vascular rupture quantified by tissue hemoglobin kits. n=6. **p<0.01; ***p<0.001, by one-way ANOVA with Bonferroni's correction.

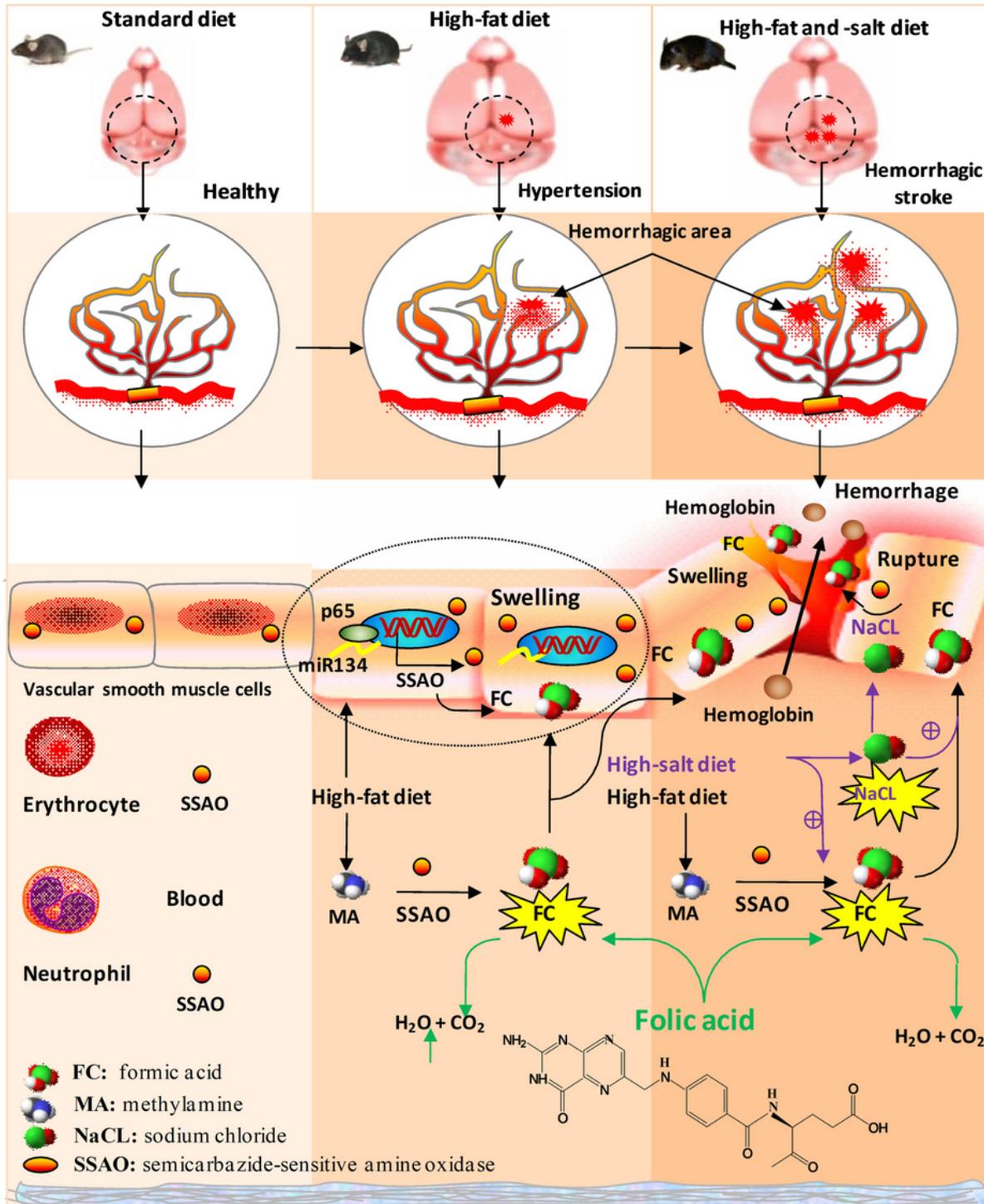


Figure 8

The enhancement of formic acid metabolism by folic acid alleviates hypertension cerebral hemorrhage. Schematic model describing the molecular mechanisms of SSAO-derived formic acid-induced hypertension cerebral hemorrhage. Folic acid (a promoting agent of FC metabolism, which transfers

formic acid to CO₂ and H₂O by activating the enzyme of THF), can prevent stroke. FC: formic acid; THF: 10-formyl tetrahydrofolate synthetase. Hemoglobin, a marker of brain vascular rupture.

Supplementary Files

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