Original research

Rapid gut dysbiosis induced by stroke exacerbates brain infarction in turn

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ABSTRACT

Objective Stroke is a leading cause of death and disability worldwide. Neuroprotective approaches have failed in clinical trials, thus warranting therapeutic innovations with alternative targets. The gut microbiota is an important contributor to many risk factors for stroke. However, the bidirectional interactions between stroke and gut microbiota remain largely unknown.

Design We performed two clinical cohort studies to capture the gut dysbiosis dynamics after stroke and their relationship with stroke prognosis. Then, we used a middle cerebral artery occlusion model to explore gut dysbiosis post-stroke in mice and address the causative relationship between acute ischaemic stroke and gut dysbiosis. Finally, we tested whether aminoguanidine, superoxide dismutase and tungstate can alleviate post-stroke brain infarction by restoring gut dysbiosis.

Results Brain ischaemia rapidly induced intestinal ischaemia and produced excessive nitrate through free radical reactions, resulting in gut dysbiosis with Enterobacteriaceae expansion. Enterobacteriaceae enrichment exacerbated brain infarction by enhancing systemic inflammation and is an independent risk factor for the primary poor outcome of patients with stroke. Administering aminoguanidine or superoxide dismutase to diminish nitrate generation or administering tungstate to inhibit nitrate respiration all resulted in suppressed Enterobacteriaceae overgrowth, reduced systemic inflammation and alleviated brain infarction. These effects were gut microbiome dependent and indicated the translational value of the brain–gut axis in stroke treatment.

Conclusions This study reveals a reciprocal relationship between stroke and gut dysbiosis. Ischaemic stroke rapidly triggers gut microbiome dysbiosis with Enterobacteriaceae overgrowth that in turn exacerbates brain infarction.

Significance of this study

What is already known about this subject?
- Ischaemic stroke is a major global health burden, and neuroprotective treatment and prevention targets are urgently required.
- Gut microbiome dysbiosis plays a critical role in the development of many stroke risk factors before the stroke event.
- The role of gut microbiota at the acute stage of stroke has not been clarified.

What are the new findings?
- Dynamic gut dysbiosis and recovery are observed in clinical cohorts of patients with ischaemic stroke. Furthermore, this dysbiosis at the acute stage is an independent risk factor for patients with poor early recovery.
- Mice with middle cerebral artery occlusion show rapid and dynamic gut dysbiosis. The driving force of this dysbiosis is stroke-induced intestinal ischaemia and reperfusion, which leads to the overgrowth of Enterobacteriaceae through nitrate respiration.
- The overgrowth of Enterobacteriaceae, in turn, accelerates systemic inflammation through the LPS-TLR4 pathway and exacerbates brain infarction.
- Diminishing nitrate generation or inhibiting nitrate respiration can suppress Enterobacteriaceae overgrowth, reduce systemic inflammation and further alleviate brain infarction.

How might it impact on clinical practice in the foreseeable future?
- Gut microbiota dysbiosis represented by Enterobacteriaceae is a potential new risk biomarker in predicting clinical outcomes for patients with acute ischaemic stroke.
- Nitrate production and respiration induced by the gut–brain axis may represent a novel treatment and prevention target, and aminoguanidine, superoxide dismutase and tungstate are promising candidates for ischaemic stroke.

INTRODUCTION

Stroke is a major global health burden with an annual incidence of 258 (95% CI 234 to 284) per 100 000 person years and 88 deaths (95% CI 80 to 94) per 100 000 person years worldwide.[1] Stroke has also become the leading cause of death and disability-adjusted life years (DALYs) in China according to the Global Burden of Diseases in 2017.[2] The high morbidity and mortality of stroke is due not only to its lethality but also to the lack of effective treatments. Recanalisation to restore brain blood flow is the major treatment approach, although this procedure requires a strict time window that is not applicable for the majority of

patients with ischaemic stroke.[3] Alternatively, neuroprotection therapies aiming to protect the ischaemic penumbra through reducing excitotoxicity, oxidative/nitrosative stress and inflammation have attracted attention,[4] although almost all of these treatments have failed in clinical trials.[5] Currently, none of the neuroprotection treatments have been recommended by the latest Guidelines for the Early Management of Patients with Acute Ischaemic Stroke.[6] Considering the intricate mechanisms underlying acute stroke progression, developing effective new treatments for stroke is an imperative challenge.

We hypothesised that the gut microbiome, which is the collective microbes inhabiting our intestinal tract, is a novel target for stroke treatment. Normally, the host maintains delicate homeostasis with its gut microbiome through immune supervision, nutrition supply and respiratory chain regulation. Disturbance of this homeostasis, namely, dysbiosis, increases stroke risk by promoting host metabolic malfunction, including obesity,[7] diabetes,[8 9] hypertension,[10 11] metabolic syndrome[12 13] and atherosclerosis.[14] Stroke may rapidly annihilate gut homeostasis as indicated by the significant gut dysbiosis observed in various stroke cohort studies[15–17] and related animal experiments.[18 19] Moreover, the gut microbiome can regulate γδT cell differentiation and affect ischaemic stroke progression through innate and adaptive immune regulation,[20 21] thus implying a reciprocally causative relationship between dysbiosis and stroke severity. Nevertheless, the bidirectional interaction between stroke and the microbiome remains largely unknown. Here, we aimed to explore the underlying mechanism of gut microbial dysbiosis induced by stroke and its effect on brain infarction. Furthermore, we suggest three potential interventions targeting gut dysbiosis for stroke treatment. Our study unravels the role of the gut–brain axis in stroke and indicates its translational value in stroke treatment.

MATERIALS AND METHODS

Study subjects

Clinical cohort 1 included 28 patients with acute ischaemic stroke and 28 healthy controls. Faecal samples were collected at T1 (0–4 days post stroke), T2 (5–7 days post stroke), T3 (8–30 days post stroke) and T4 (1–4 months post stroke) for patients with acute ischaemic stroke. Clinical cohort 2 included 124 patients with acute ischaemic stroke. Their faecal samples were collected within 48 hours after admission, and the patients' clinical outcomes were documented. The gut microbiome was profiled by sequencing the faecal 16S rRNA gene.

Animal experimental protocol

A middle cerebral artery occlusion (MCAO) model was used as the mouse stroke model. For the bacterium transplantation experiment, mice were treated with an antibiotics combination (vancomycin, gentamicin, metronidazole and ampicillin) for 1 week,[20] followed by a 1 day washout period, and were gavaged with 200 µL of phosphate buffered saline suspended bacteria (OD=1) each day for another week until MCAO was conducted. Aminoguanidine (AG) and sodium tungstate (W) were obtained from Sigma-Aldrich. Superoxide dismutase (SOD) and SOD^{H84A} were obtained from Hangzhou Redox Pharmatech Co. (Hangzhou, China).[22]

Statistical analysis

Statistical analyses were performed using R3.5.1 and SPSS 20.0 (IBM, USA). Data were expressed as the median (IQR) and were analysed by the Wilcoxon rank sum test. Univariate and multivariate logistic regression analyses were performed, and ORs and 95% CIs were calculated to identify the risk factors for stroke outcome. Medians±quartiles are plotted in all box plots.

Other materials and methods are described in the online supplemental materials and methods.

RESULTS

Enterobacteriaceae enrichment is an independent risk factor for patients with acute ischaemic stroke in early stage recovery

We recruited two independent cohorts of patients with ischaemic stroke to explore the dynamics of gut microbiome dysbiosis and its correlation with stroke outcome. In the first cohort, 28 patients with ischaemic stroke were enrolled and a series of faecal samples from patients in the acute (T1, T2), subacute (T3) and convalescent (T4) phases were collected (online supplemental table S1, online supplemental figure S1A). According to principal coordinate analysis (PCoA) based on the weighted UniFrac distance, the microbiome structures at the acute (T1 and T2) and subacute phases (T3) were clearly separated from those in age-matched and sex-matched healthy controls and recovered at the convalescent phase (T4) (figure 1A,B). Using QIIME2, 97% of sequences were classified to the family level, which decreased to 67% at the genus level due to the limited resolution of the 16S rRNA gene tag (online supplemental figure S1B). At the family level, Enterobacteriaceae, Ruminococcaceae, Veillonellaceae and Lachnospiraceae were significantly enriched while Bacteroidaceae and Prevotellaceae were markedly depleted after stroke (figure 1C and online supplemental figure S1C). Among them, Enterobacteriaceae showed the highest increase after stroke and recovery in T4, which was similar to the whole-community dynamics (figure 1C,D), while Bacteroidaceae displayed the opposite dynamic trend (figure 1C and online supplemental figure S1D).

In the second cohort, we recruited 124 patients with ischaemic stroke, collected their first faecal samples within 48 hours of admission, and evaluated their primary outcomes at 7 days post treatment. These patients were classified as having either poor (<40% improvement, n=72) or good primary outcomes (≥40% improvement, n=52) based on their National Institutes of Health Stroke Scale (NIHSS) score improvement at 7 days compared with that at baseline (online supplemental table S2, online supplemental figure S1F). These two groups did not show significant differences in their initial NIHSS scores on admission (online supplemental figure S1H). The microbiome analysis showed that many of the relative abundances of the stroke-related taxa identified above were not significantly different between these two groups (figure 1E and online supplemental figure S1G,I–O) except for that of Enterobacteriaceae, which was significantly enriched in the poor primary outcome group (figure 1E,F). We then tested whether Enterobacteriaceae is an independent risk factor for stroke patients with poor primary outcomes. Among all the collected clinical data, homocysteine, which is a risk factor for long-term mortality of patients with ischaemic stroke,[23] was significant in the univariate logistic regression analysis (figure 1G) but was not significant in the multivariate logistic regression analysis (figure 1H). Conversely, Enterobacteriaceae enrichment remained significant in both the univariate (OR 1.72, 95% CI 1.31 to 2.27) and multivariate logistic regression analyses (OR 1.97, 95% CI 1.23 to 3.16), indicating that Enterobacteriaceae is an independent risk factor for stroke patients with poor primary outcomes.

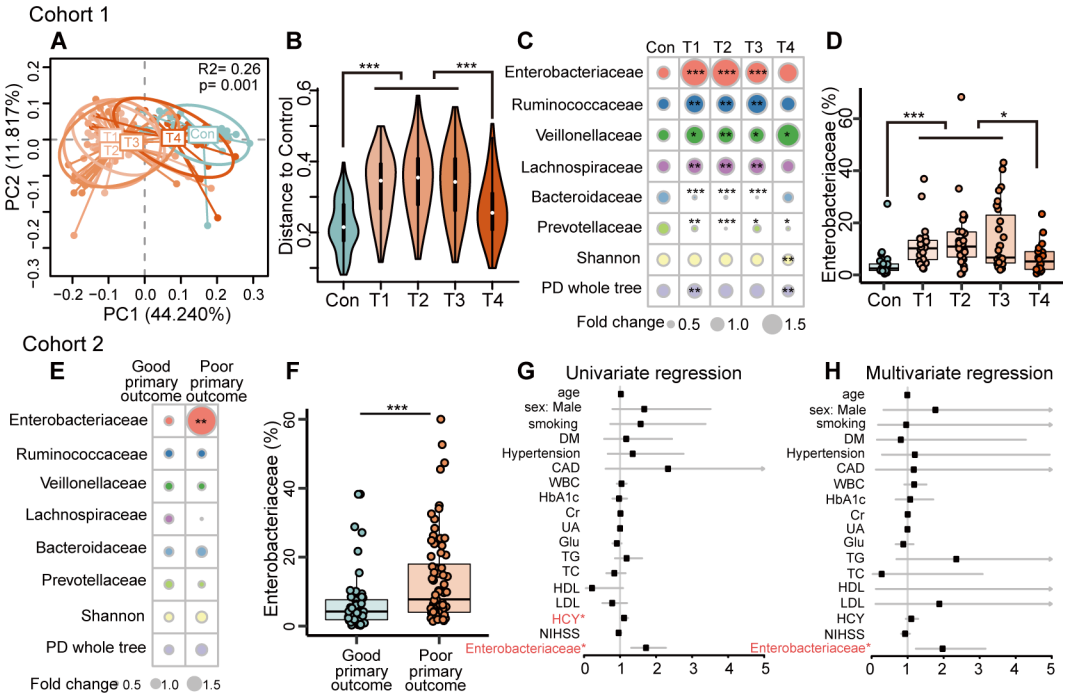

Figure 1 Gut microbiota changes dynamically with the prolongation of stroke, and Enterobacteriaceae is an independent risk factor. (A)–(D) Clinical cohort 1 included 28 patients with ischaemic stroke and 28 healthy controls. (A) Principal coordinate analysis showing the gut microbiota composition among healthy controls and the acute (T1–T2), subacute (T3) and convalescent (T4) phases of patients with ischaemic stroke. (B) Distance to the control group among all ischaemic stroke groups. (C) Bubble chart distributing significantly different bacterial families and gut microbial α diversity (Shannon and PD whole tree index) among the control group and all ischaemic stroke groups. (D) Relative abundance of Enterobacteriaceae among the control group and all ischaemic stroke groups. (E)–(H) Clinical cohort 2 included 124 patients with ischaemic stroke with either good or poor outcomes. (E) Bubble chart distributing significantly different taxa between the good and poor primary outcome groups. (F) Relative abundance of Enterobacteriaceae was significantly higher in the poor primary outcome group. (G) Univariate logistic regression analysis of the risk factors for a poor primary outcome of patients with ischaemic stroke. (H) Multivariate logistic regression of the risk factors for a poor primary outcome of patients with ischaemic stroke after adjusting for confounding factors. Medians±quartiles are plotted; *p<0.05, **p<0.01 and ***p<0.001 by the Wilcoxon RANK sum test. CAD, coronary artery disease; Cr, creatinine; DM, diabetes mellitus; Glu, glucose; HbA1c, hemoglobin A1c; HCY, homocysteine; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NIHSS, national institutes of health stroke scale; TC, total cholesterol; TG, triglycerides; UA, uric acid; WBC, white blood cell.

Ischaemic stroke induced rapid gut dysbiosis with Enterobacteriaceae blooming

After identifying gut microbiome dysbiosis with Enterobacteriaceae enrichment in patients with acute stroke, we sought to determine whether this dysbiosis was caused by stroke occurrence. We used MCAO, a classic stroke model, to explore whether the stroke event could induce significant gut microbiome dysbiosis (figure 2A). To determine the gut microbiome changes after stroke, we conducted MCAO or a sham operation and sacrificed the mice at 3 hours, 6 hours, 12 hours, 1 day, 3 days and 7 days post stroke for dynamics tracking. According to our results, artery occlusion successfully induced brain infarction as early as 3 hours post stroke, with a significant increase beginning at 12 hours and peaking at 1 day until 7 days (figure 2B,C).

Interestingly, a dimension reduction method showed that the gut microbiome presented clear and rapid dynamic changes in accordance with increasing brain infarctions in principal coordinate one (PC1) and PC3. As early as 3 hours post stroke, the ileum content microbiome showed obvious deviation from that of the control group (figure 2D,E). The microbiome reached its maximal deviation from 12 hours to 1 day after stroke and then gradually recovered to a state similar to that of the control group at 7 days post stroke. This deviation-recovery pattern in mice was similar to the trend in patients with stroke but within a much shorter time period (figure 1A). Similarly, we found that Enterobacteriaceae was the most significantly changed taxon after stroke modelling (linear discriminant analysis=4) (online supplemental figure S2A). The relative Enterobacteriaceae abundance increased from less than 1% at normal status to higher than 10% as early as 6 hours post-stroke, and it peaked as high as 40% from 12 hours to 1 day and gradually recovered from 3 days to 7 days post stroke in the ileum (figure 2F and H). The dynamic change occurred in not only the contents of the ileum but also in the contents of three additional gastrointestinal sections, that is, the jejunum, cecum and colon (figure 2G, I,J, and online supplemental figure S2B–J). In addition to 16S rRNA sequencing, we performed quantitative PCR with *Escherichia coli* specific primers and confirmed significant overgrowth of the bacteria from hundreds to tens of thousands of fold in the four gastrointestinal sections at 1 day post stroke in the MCAO group compared with a 1 day time point in the sham group (online supplemental figure S2K–N).

Gut dysbiosis is associated with stroke-induced intestinal ischaemia and nitrate production

To explore the underlying mechanism of rapid gut dysbiosis induced by stroke, we compared the gene expression profiles of mouse cecal tissue using RNA sequencing between the MCAO and sham groups. We found that only the glutathione metabolic

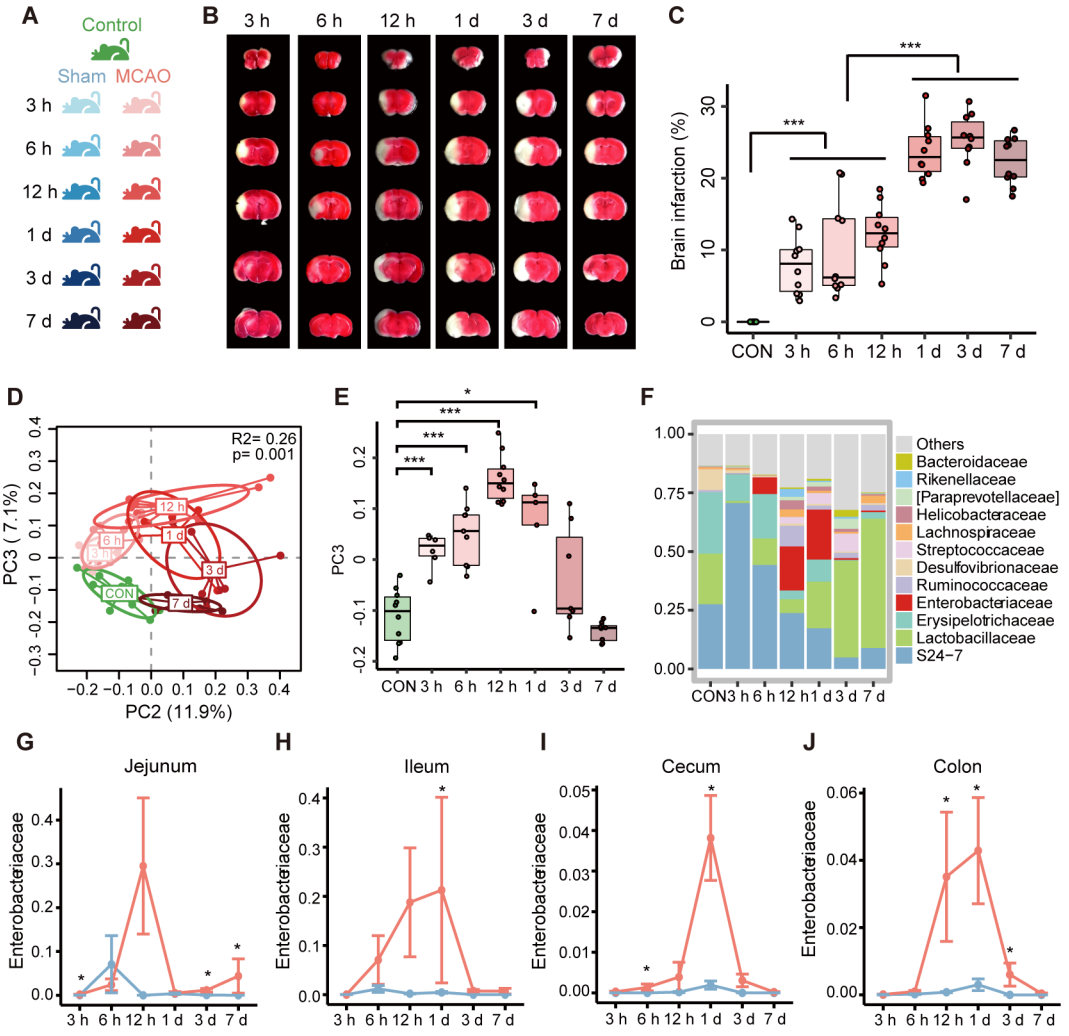

Figure 2 Middle cerebral artery occlusion (MCAO) mice exhibit rapid gut microbiota dysbiosis with significant enrichment in Enterobacteriaceae. (A) Animal experiment design for the control group (n=10 mice), sham groups (mice were divided into different groups based on when they were sacrificed, and their samples were collected at 3 hours, 6 hours, 12 hours, 1 day, 3 days and 7 days post sham operation, n=5 mice/time point), and MCAO groups (mice were divided into different groups based on when they were sacrificed, and their samples were collected at 3 hours, 6 hours, 12 hours, 1 day, 3 days and 7 days post MCAO, n=10 mice/time point). (B) Brain sections of all MCAO groups. (C) Related brain infarction ratio of the control group and all MCAO groups. (D)–(F) Temporal shifts of the gut microbiota in ileum content samples from the control group and all MCAO groups, including principal coordinate analysis distribution based on unweighted UniFrac distances (D), time series analysis of the community structure on principal coordinate 3 (PC3) (E), and gut microbial composition at the family level (F). (G)–(J) Temporal shifts in relative Enterobacteriaceae abundance using 16S rRNA sequencing between the sham and MCAO groups in jejunum (G), ileum (H), cecum (I) and colon (J) sections at all time points. Medians±quartiles are plotted; *p<0.05, **p<0.01 and ***p<0.001 by the Wilcoxon RANK sum test.

process pathway was significantly upregulated in the MCAO group according to the pathway analysis (online supplemental figure S4A, B). Since glutathione is involved in the process in oxidative stress and oxidative stress is mainly triggered by ischaemia and reperfusion,[24 25] we hypothesised that the gastrointestinal tract also experienced ischaemia accompanying stroke. To test this hypothesis, we used a laser speckle imaging system to detect the blood flow in the mouse cecum during MCAO. When removing filaments from the carotid artery, the blood flow in the mouse cecum was significantly reduced compared with that in the sham group, which persisted for 1 hour (figure 3A,B), thus supporting our hypothesis that the gastrointestinal tract also experienced ischaemia accompanying stroke (figure 3C). We further measured the expression of three critical genes during ischaemia-reperfusion injury: *Nox1* and *Duox2* for superoxide production[26 27] and *Nos2* for nitric oxide (NO) production.[28] All three genes were significantly upregulated at 1 day post stroke compared with those in the sham group, which further supports the existence of intestinal ischaemia-reperfusion injury during stroke (figure 3D–F). Additionally, their relative expression levels also showed a temporal shift pattern, with an increasing trend beginning at 3 hours and a peak value approximately 1 day to 3 days post stroke (online supplemental figure S4C–E). Superoxide and NO could rapidly react with each other and produce cytotoxic peroxynitrite, which would further spontaneously convert to nitrate, a chemical known to boost Enterobacteriaceae overgrowth in an anaerobic microenvironment through nitrate respiration.[29 30] Herein, we determined the nitrate concentration in the mouse cecum mucus layer and observed a significant increase from 9.12 µM at 3 hours to 105.50 µM at 1 day post stroke (online supplemental figure S4F), which was also significantly higher than the 16.98 µM concentration in the sham group (figure 3G). The level of nitrate was sufficient to induce the overgrowth of Enterobacteriaceae as shown by the

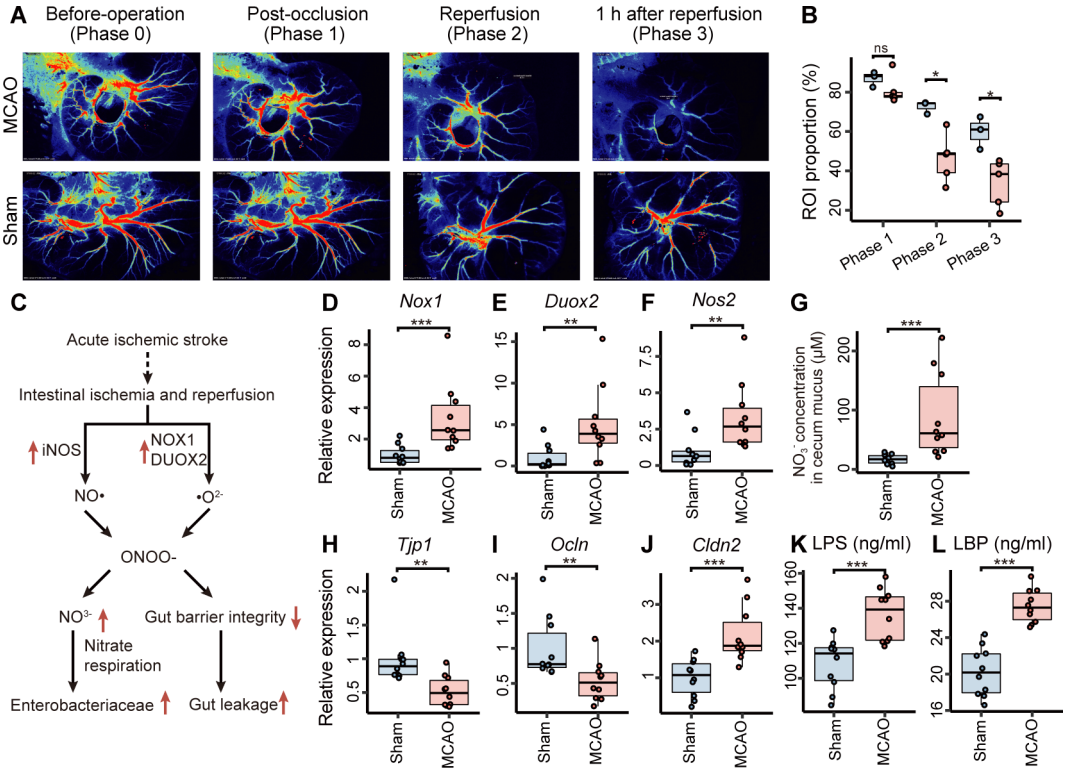

Figure 3 Gut microbial dysbiosis is induced by nitrate respiration after intestinal ischaemia and reperfusion in middle cerebral artery occlusion (MCAO) mice. (A) Laser speckle images and (B) related ROI proportions of mouse cecum blood flow between the sham group (n=3) and MCAO group (n=5) during sham and MCAO operations. (C) Diagram of the proposed mechanism of intestinal ischaemia and reperfusion-induced free radical reaction post stroke. Relative (D) *Nox1*, (E) *Duox2* and (F) *Nos2* expression; (G) nitrate concentration in the mouse cecum mucus layer; relative (H) *Tjp1*, (I) *Ocln* and (J) *Cldn2* expression; and (K) serum lipopolysaccharide (LPS) and (L) LPS binding protein (LBP) levels in the sham and MCAO groups at the 1 day time point (n=10 mice/group). Medians±quartiles are plotted; *p<0.05, **p<0.01, and ***p<0.001 by the Wilcoxon RANK sum test. DUOX2, dual oxidase 2; iNOS, inducible nitric oxide synthase; NOX1, NADPH oxidase 1.

supplementation of 0–200 μM nitrate in the gut microbiome in vitro (online supplemental figure S4G). It is noteworthy that the elevated *Nox1* and *Duox2* expression, increased nitrate concentration, Enterobacteriaceae overgrowth, and brain infarction increase all consistently peaked at 1 day post stroke.

In addition to gut microbiome dysbiosis, intestinal ischaemia-reperfusion injury and peroxynitrite production also impair the gut barrier integrity.[31] We determined the relative expression of tight junction protein genes, including *Tjp1*, *Ocln* and *Cldn2*, and found significantly decreased *Tjp1* and *Ocln* expression and significantly elevated *Cldn2* expression in the MCAO group at 1 day post stroke compared with those in the related sham group (figure 3H–J). In addition to the disrupted gut barrier, gut leakage biomarkers in mouse serum were also determined. Our results showed that lipopolysaccharide (LPS), LPS- binding protein (LBP) and D-lactose (D-lac) were significantly enriched in the MCAO group (figure 3K,L, and online supplemental figure S4H), and they showed a similar temporal shift pattern that peaked at 1 day post stroke (online supplemental figure S4I–K).

Enterobacteriaceae exacerbates brain infarction by accelerating systemic inflammation

Since the rapid intestinal Enterobacteriaceae blooming was temporally consistent with increasing brain infarction, we wondered whether Enterobacteriaceae overgrowth played a role in the pathophysiological process of stroke. As a typical Enterobacteriaceae species, *Escherichia coli* showed significant overgrowth in both the male and female mice stroke model, and we sought to determine whether *E. coli* can affect stroke progression. Our results showed that inoculating an *E. coli* strain isolated from MCAO-treated mice into antibiotic-treated mice significantly increased brain infarction compared with that in the corresponding antibiotic only treated mice (Abx group) (figure 4A,B). To explore the underlying mechanism, we compared differentially expressed genes of mouse cecum tissue between antibiotic and *E. coli* treated groups through RNA sequencing and found that genes involved in immune system processes, the innate immune response and the response to cytokines based on Gene Ontology (GO) terms were significantly enriched in the *E. coli* treated group (figure 4C). According to the KEGG analysis, upregulated pathways in the *E. coli* treated group included interferon signalling, NOD-like receptor signalling, RIG-I-like receptor signalling, and toll-like receptor (TLR) signalling. Therefore, we further determined the levels of systemic inflammation related biomarkers in mouse serum and found that the LPS, LBP, TNF-α, interleukin (IL)-1β,and IL-6 concentrations were significantly higher in the *E. coli* treated group than in the Abx group, thus indicating that increased *E. coli* abundance in the gut microbiome may accelerate systemic inflammation after stroke (figure 4D–H). In addition, our results showed that TLR4 pathway related genes, including *Tlr4*, *Traf6*, *Tram*, *Myd88* and *Nfkb*, were significantly upregulated in *E. coli* inoculated mice (figure 4I–M). To verify the role of the LPS-TLR4 pathway,[32] we knocked out the *waaL* gene, which is involved in the biosynthetic pathways of integral LPS.[33] The *E. coli waaL*

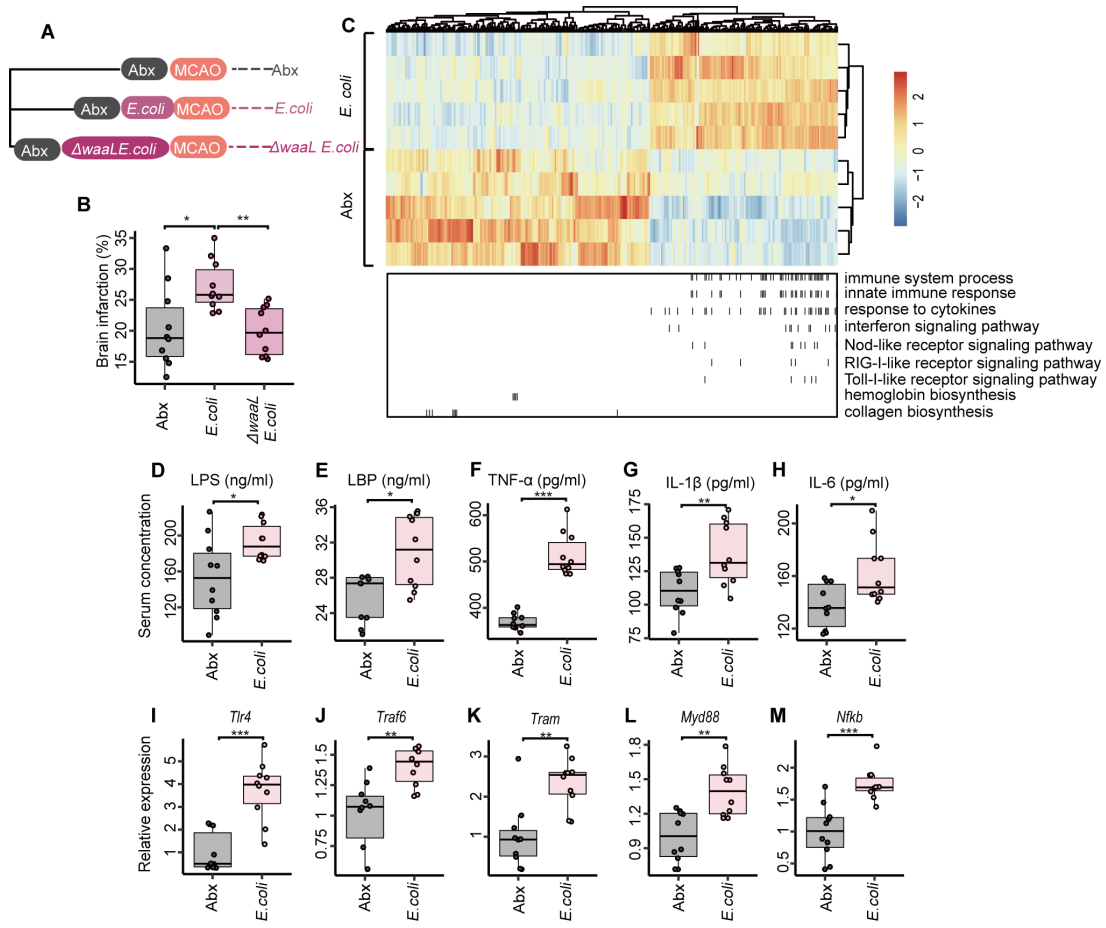

Figure 4 Enterobacteriaceae overgrowth promotes brain infarction. (A) Animal experiment design and (B) brain infarction ratios of the Abx group, *Escherichia coli* group, and Δ*waaL E. coli* group (n=10 mice/group). (C) Heatmap based on RNA sequencing analysis of cecum tissue from the *E. coli* group and Abx group revealed significantly altered gene expression between the two groups (n=5 mice/group). Genes involved in the immune and systemic inflammation pathways are annotated. (D) Serum lipopolysaccharide (LPS), (E) LPS binding protein (LBP), (F) tumour necrosis factor (TNF)-α, (G) interleukin (IL)-1β, and (H) IL-6 levels between the Abx group and the *E. coli* group. Relative (I) *Tlr4*, (J) *Traf6*, (K) *Tram*, (L) *Myd88* and (M) *Nfkb* expression between the Abx group and the *E. coli* group. Medians±quartiles are plotted; *p<0.05, **p<0.01 and ***p<0.001 by the Wilcoxon RANK sum test.

mutant inoculated group showed a brain infarction ratio comparable to that of the Abx group, which was significantly lower than that of the *E. coli* treated group (figure 4A,B). In addition, another *E. coli* strain isolated from a patient with acute ischaemic stroke (*E. coli* AIS01) also exacerbated brain infarction in the mice model (online supplemental figure S4L). These data suggest that *E. coli* overgrowth in the gut may exacerbate brain infarction through LPS-mediated systemic inflammation.

Inhibiting Enterobacteriaceae overgrowth alleviates brain infarction

To test the potential role of Enterobacteriaceae overgrowth as a novel target for stroke treatment, we used three inhibitors targeting different pathways: AG to inhibit iNOS-mediated NO production,[34] a heat stable Mn-type SOD to eliminate superoxide,[35] and sodium tungstate (W) to inhibit the nitrate respiration of Enterobacteriaceae.[36] All three materials were orally administered at the 1 hour time point post MCAO (figure 5A). As expected, the nitrate concentrations in the cecum mucus layer of both the AG and SOD groups were comparable to that of the sham group and were remarkably lower than that of the MCAO group (figure 5B). Interestingly, the inhibitors not only inhibited free radical production but also significantly inhibited the related gene expression at the transcriptional level. The relative *Nos2* expression was significantly decreased in the AG group, and *Nox1* and *Duox2* expression was significantly reduced in the SOD group (figure 5C–E), suggesting the possible positive feedback regulation of iNOS.[37]

AG, SOD and W significantly restored gut dysbiosis according to PCoA (figure 5F–H) and reduced Enterobacteriaceae abundance to the sham group level (figure 5I–K), supporting the fact that nitrate respiration was the major driving force of gut dysbiosis after stroke. More importantly, AG, SOD and W significantly reduced the brain infarction ratio (figure 5L–N) as well as rescued hippocampal neurons (online supplemental figure S5A), further confirming that gut dysbiosis was crucial for the progression of post-stroke impairment.

Although AG, SOD and W rescued gut dysbiosis and reduced brain infarction, it was still unclear whether this improvement in brain infarction was mediated by the gut microbiome. Therefore, we treated the mice with broad-spectrum antibiotic cocktails before MCAO and then treated the mice with AG (Abx-AG), SOD (Abx-SOD) or W (Abx-W) after MCAO. Antibiotics obviously attenuated the protective effects of these three approaches, as the brain infarctions in the Abx-AG, Abx-SOD and Abx-W groups showed no significant differences compared with those

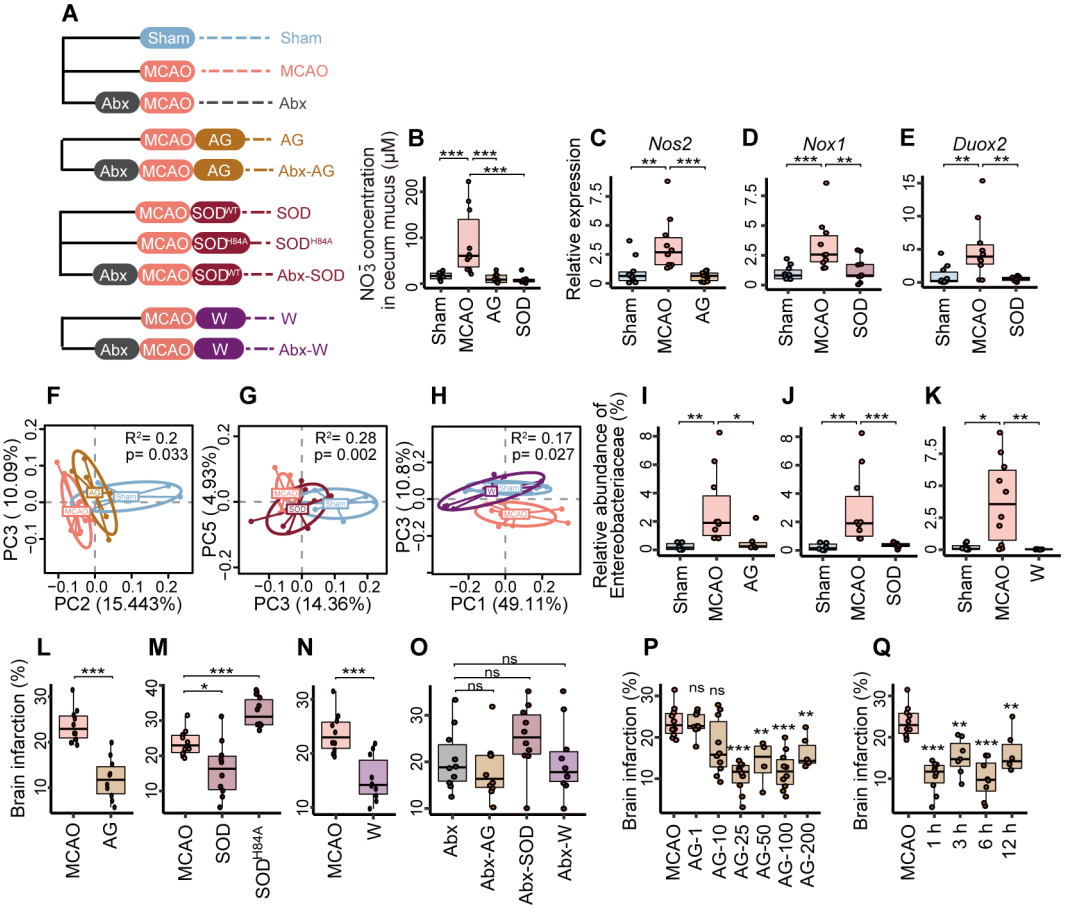

Figure 5 Inhibiting Enterobacteriaceae overgrowth by administrating aminoguanidine (AG), superoxide dismutase (SOD) or sodium tungstate (W) improves brain infarction. (A) Animal experiment design of Ag, SOD and W intervention (n=10 mice/group). (B) Nitrate concentration in the mouse cecum mucus layer among the sham group, middle cerebral artery occlusion (MCAO) group, AG group and SOD group. (C) Relative *Nos2* expression among the sham group, MCAO group and AG group. Relative (D) *Nox1* and (E) *Duox2* expression among the sham group, MCAO group and SOD group. Principal cooridinate analysis showing the cecum content distribution among the sham group, MCAO group, and (F) AG, (G) SOD or (H) W group. Relative Enterobacteriaceae abundance in the cecum contents among the sham group, MCAO group, and (I) AG, (J) SOD or (K) W group. (L)–(N) Brain infarction ratios for the AG, SOD or W intervention. Brain infarction ratios between the MCAO group and AG group (L); among the MCAO group, SOD group and SOD^{H84A} group (M); and between the MCAO group and W group (N). (O) Brain infarction ratios among the Abx group, Abx-AG group, Abx-SOD group and Abx-W group. (P) Brain infarction ratios for AG administered at 1, 10, 25, 50, 100 and 200 mg/kg at 1 hour post MCAO (n=4–10 mice/group). (Q) Brain infarction ratio for AG administered with 25 mg/kg at 1 hour, 3 hours, 6 hours and 12 hours post MCAO (n=6–10 mice/group). Medians±quartiles are plotted; *p<0.05, **p<0.01 and ***p<0.001 by the Wilcoxon RANK sum test.

of the Abx group (figure 5O), suggesting that the gut microbiome was necessary for AG, SOD and W mediated improvement of brain infarction during stroke.

To demonstrate whether the effect of SOD was achieved by its superoxide dismutation activity in the intestinal microenvironment, we constructed a SOD^{H84A} mutant in which the amino acid histidine at the active centre was mutated to alanine. This mutant enzyme lacks Mn-binding activity and is therefore unable to dismutate superoxide. Compared with the MCAO group, the SOD^{H84A} group did not ameliorate brain infarction (figure 5M).

AG, SOD or W administration restored gut barrier dysfunction as demonstrated by the LPS, LBP, D-lac and malonaldehyde (MDA) results (online supplemental figure S5J–L, S6I-K, and S7I,J). An analysis of the relative expression of tight junction protein genes including *Cldn2*, *Tjp1* and *Ocln* further supports the above observation (online supplemental figure S5O–Q, S6N-P, and S7M, N). Moreover, these approaches alleviated systemic inflammation, which was indicated by significantly decreased serum TNF-α and IL-6 concentrations and expression of proinflammatory cytokine genes, including *Tnf*, *Il6*, *Il1b*, *Kc* and *Cxcl2* (online supplemental figure S5M, N, R–V, S6L, M, Q–U, and S7K, L, O–Q). As markers of Th1 and Th17 T cell polarisation, the proinflammatory cytokines interferon γ (IFN-γ) and IL-17 are associated with worse outcomes in mouse models of stroke.[21] We found that relative *Ifng* expression was significantly reduced in AG, SOD and W treated mice, and that *Il17* expression was reduced in AG and SOD treated mice (online supplemental figure S5W, X, S6V, W, and S7R).

As a promising therapeutic approach to ameliorate brain infarction, we further tested the dose-response relationship of AG targeting stroke. When AG was administered at 1 hour post stroke, the effective AG administration dose ranged from 25 to 200 mg/kg, with the 25 mg/kg dose showing the best performance (figure 5P). Compared with other effective doses, 25 mg/kg showed the strongest effect in suppressing Enterobacteriaceae overgrowth (online supplemental figure S5Y,Z). Furthermore, we tested the therapeutic time window by administering 25 mg/kg AG to the MCAO model mice and found that AG significantly reduced brain infarction from 1 hour until 12 hours post stroke (figure 5Q). The fact that the Enterobacteriaceae abundance in

the MCAO group was still significantly higher than that in the related sham group at 12 hours post stroke (figure 2F–J) may help explain the efficacious therapeutic effects of AG administered at 12 hours post stroke.

DISCUSSION

To our best knowledge, this is the first study revealing not only the aetiological role of gut dysbiosis in a critical disease but also the driving force of this dysbiosis under a disease state, thus demonstrating a reciprocal causative relationship between them. During the past decade, gut dysbiosis was found to cause many diseases ranging from metabolic disorders to central nervous system diseases,[7–11 13 14 18–20 38 39] although the driving force of dysbiosis remains largely elusive. As observed, the gut microbiome of patients with ischaemic stroke from clinical cohorts was dysbiotic. This dysbiosis, at least partially, was rapidly induced by the stroke event. At 3 hours after MCAO modelling, our results demonstrated a consistent temporal shift pattern that included the expression of ischaemic reperfusion genes, the deviation of the gut microbiome from the normal status with the rapid overgrowth of Enterobacteriaceae, and the increase of brain infarction. Although concerns about the possibility of intestinal ischaemia after stroke have been raised, the present study provides imaging and molecular evidence of this pathology. The intestinal ischaemia-reperfusion finally drove the gut dysbiosis due to the nitrate respiration of the gut microbiota at all four intestinal sections. Our results not only revealed a novel pathway of the brain to gut interaction but also provided a series of potential new targets for the treatment and prevention of stroke.

The growth of Enterobacteriaceae in the intestines along with the impaired gut barrier rapidly induced systemic inflammation through the production of a large amount of LPS. The increases in LPS and LBP in the plasma of stroke patients were observed, and higher levels of LPS and LBP were associated with worse outcomes in patients with ischaemic stroke.[40 41] In addition, LPS injection exacerbated neurological deficit and impaired survival after experimental stroke.[42] Here, we observed that LPS may contribute to the exacerbation of brain infarction by accelerating the inflammation after stroke under significantly elevated TNF-α and IL-6 levels in mice sera. These inflammatory cytokines were reported to increase in patients with ischaemic stroke and exacerbate brain ischaemic injury.[43–45] Nevertheless, whether this increase in LPS is a short-term effect from the mushrooming of Enterobacteriaceae after stroke attack has not been clarified. Our results showed that inhibiting the overgrowth of Enterobacteriaceae induced by intestinal ischaemia-reperfusion reduced systemic inflammation and brain infarctions. Moreover, the relative abundance of Enterobacteriaceae was an independent risk factor for the early recovery of patients with ischaemic stroke, suggesting that the gut microbiome played a role at the acute stage of the stroke event. Although Enterobacteriaceae was the only LPS-producing bacteria family found to be associated with stroke outcome, other bacteria may also serve as a resource in producing LPS, which may function in the LPS-TLR4-systemic inflammation pathway during stroke. Future studies employing high-resolution techniques are needed to characterise the stroke-related dysbiosis pathogens at lower taxonomic levels. Although intestinal effector T cells may traffic to leptomeninges to enhance ischaemic neuroinflammation post stroke via the secretion of IL-17,[20] our study revealed that rapid gut dysbiosis within 24 hours post stroke may promote brain infarction through systemic inflammation. Inflammatory responses have been a therapeutic target for patients with acute ischaemic stroke, although most of these neuroprotection treatments have failed.[5] Our study suggests that pathogen-associated molecular patterns from gut dysbiosis could be a critical resource of the inflammation and therefore may represent an important target for the neuroprotection of stroke treatment.

Three inhibitors not only provide evidence for the molecular mechanism underlying the crosstalk relationship between stroke and gut dysbiosis but also highlight potential translational values. AG was well known for inhibiting iNOS activity, and it was developed as an advanced glycation end product inhibitor targeting α-dicarbonyl compounds in diabetes treatment.[46] However, it failed in a phase II clinical trial (ACTION II) due to the side effects of a dosage of 600 mg/day for two consecutive years.[47] Our results indicated that AG has potential uses at the acute stage of stroke at a relatively low dosage and applicable time window. In addition to AG, SOD is a promising candidate as well. Our results demonstrate that this specific heat-stable SOD could function in the gastrointestinal tract through oral administration. As a protein enzyme, the detailed mechanism maintains SOD activity in the gut and the associated metabolism deserves further study.

Moreover, the gut dysbiosis post stroke observed in our study might be a mixture of dysbiosis induced by the stroke event as well as associated metabolic diseases or post-admission treatment. Future studies tracing populations with a low stroke risk until the occurrence of stroke events might be able to disentangle the gut dysbiosis induced by the stroke event from other influencing factors. However, our results indicated that the current treatment for patients with stroke fails to restore gut dysbiosis, which is a potential new therapeutic target linking brain infarction exacerbation and poor outcomes for patients with stroke.

Although we provided the first dynamic imaging evidence of intestinal ischaemia after stroke, the present study did not elucidate the mechanism underlying this linkage. We deduced that stroke may induce intestinal ischaemia-reperfusion through the sympathetic nervous system because the blockage of β-adrenergic receptors significantly reduces gut permeability and norepinephrine release modifies gut microbiota dysbiosis post stroke.[18 48] In addition to stroke, many other diseases may stimulate the sympathetic nervous system, such as inflammatory bowel disease,[49] arrhythmia[50] and ischaemic heart disease,[51] and they may cause intestinal ischaemia and result in nitrate production and Enterobacteriaceae overgrowth. This finding implies that the application of the therapeutic targets mentioned in our study, such as nitrate production, nitrate respiration and Enterobacteriaceae overgrowth, could be further expanded. Further studies on whether similar brain–gut crosstalk-involved diseases occur and the contribution of gut dysbiosis to these diseases are warranted.

Contributors KX, YH, JY and HZ designed the study. KX, XG, GX, MC, NZ, CY and XT performed and supervised the human experiments. KX, XG, GX, SW, HD, HW, XZ, CT, FM and HL performed and supervised the animal experiments. KX, YH, PL and WT performed and analyzed all the data. KX, YH and HZ wrote the manuscript. KX, YH, JY and HZ conceived the study, supervised the participants, and revised the manuscript.

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