Hyperglycemia primes NETosis, which exacerbates ischemic brain damage

Jiangshan Deng (✉ johnson120@126.com)

Shanghai Jiao Tong University Affiliated Six People's Hospital <https://orcid.org/0000-0002-2996-5979>

Fei Zhao

Shanghai Jiao Tong University Affiliated Six People's Hospital

Yunlong Zhang

Shanghai Jiao Tong University Affiliated Six People's Hospital

Yajun Zhou

Shanghai Jiao Tong University Affiliated Six People's Hospital

Xiaofeng Xu

Shanghai Jiao Tong University Affiliated Six People's Hospital

Xiaojie Zhang

Shanghai Jiao Tong University Affiliated Sixth People's Hospital

Feng Wang

Shanghai Jiao Tong University Affiliated Sixth People's Hospital

Yuwu Zhao

Shanghai Jiao Tong University Affiliated Sixth People's Hospital

Research

Keywords: middle cerebral artery infarction, diabetes, hyperglycemia, neutrophil extracellular traps

Posted Date: December 4th, 2019

DOI: <https://doi.org/10.21203/rs.2.18044/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background Hyperglycemia is common and associated with poor outcomes in acute ischemic stroke patients. It is not well understood how hyperglycemia exacerbates brain damage in ischemic stroke. Neutrophil extracellular traps (NETs) have shown an emerging role in noninfectious diseases. We aimed to determine the role of NETs in acute ischemic stroke with hyperglycemia.

Methods: NETs were immunostained using NET markers (citrullinated histone H3 (H3Cit)) and quantified in thrombi retrieved from ischemic stroke patients undergoing endovascular treatment. BKS-db/db and wild-type mice were used to establish the permanent middle cerebral artery occlusion (pMCAO) model. Wild-type mice were injected with glucose to simulate acute hyperglycemia after middle cerebral artery occlusion. NETs were detected in the peri-ischemic brain tissue. After inhibition of NET formation, infarction volume, neurological function and inflammatory factors in pMCAO mice were evaluated.

Results: H3Cit, a marker of NETs, was observed in almost all thrombi. H3Cit was much more abundant in thrombi from diagnosed diabetic patients and acute hyperglycemic patients compared with those in normglycemic patients. In pMCAO mice, NETs were induced by chronic diabetes and acute hyperglycemia. Inhibition of NET formation with the peptidylarginine deiminase 4 (PAD4) inhibitor Cl-amidine decreased the infarction volume both in db/db and wild-type mice with hyperglycemia. Neurological function deficits were alleviated by blocking NET formation, as shown in the grip strength and rotarod tests. The levels of TNF- α and IL-1 β but not IL-6 coincided with NET formation.

Conclusions: Hyperglycemia may exacerbate brain damage in ischemic stroke through NETs. The underlying mechanisms deserve to be further studied.

Background

Stroke is the leading cause of disability and mortality worldwide. Hyperglycemia arises in nearly 40% of people with acute ischemic stroke[1]. Most of these individuals have diagnosed or pre-existing but unrecognized diabetes mellitus, and some nondiabetic patients may have acute stress hyperglycemia[2]. Compared to the risks in stroke patients with normoglycemia, hyperglycemic stroke patients have a much higher risk of short-term mortality and poor functional recovery, especially in nondiabetic stroke patients[3]. Additionally, many studies have shown that hyperglycemia is associated with symptomatic hemorrhagic transformation, infarct volume growth and worse clinical outcomes among patients treated with intravenous recombinant human tissue plasminogen activator (rtPA) and mechanical thrombectomy[4–9]. These observational studies consistently found an association between hyperglycemia and worse outcomes in acute ischemic stroke, but it has not been determined whether this is a cause-and-effect relationship. Blood glucose lowering treatments have failed to benefit ischemic stroke patients until now[10]. Mechanisms by which hyperglycemia exacerbates ischemic brain damage need to be explored. Recently, the emerging role of neutrophil extracellular traps (NETs) has been observed in many kinds of noninfectious diseases[11], including atherosclerosis and

atherothrombosis[12]. Early reports demonstrated that NET formation is strictly dependent on glucose and is increased in diabetes[13–15]. Hyperglycemia alone in the presence or absence of diabetes in vivo and high glucose concentration in vitro increase NETosis[16]. In addition, levels of the NET markers citrullinated H3 (citH3), cell-free DNA (cfDNA), and nucleosomes were elevated in the plasma of stroke patients[17]. NETs were also found in ischemic stroke thrombi retrieved from patient occluded arteries[18] and the plasma of ischemic stroke patients, which may constitute a useful prognostic marker in patients with acute stroke [17].

In this study, we aimed to offer solid experimental evidence of hyperglycemia-mediated exacerbation of ischemic brain damage that is mediated by NETs.

Methods

Patient thrombi collection and processing

Thrombi were collected from acute ischemic stroke patients after mechanical thrombectomy that was performed at Shanghai Jiaotong University Affiliated Sixth People's Hospital. All patients gave written informed consent under the approval of the Ethics Committee. Clinical patient data were collected, such as random glucose at admission and fasting and postprandial blood glucose the day after admission. Forty-one thrombi were available for histological analysis. Patients were divided into three groups: normoglycemia (NG), diabetes mellitus (DM) and acute hyperglycemia (HG). Acute hyperglycemia was defined as HbA1c<6.5%, fasting blood glucose $\geq$ 7.0 mmol/L or random blood glucose $\geq$ 11.1 mmol/L.

Animals

Male BKS-db/db mice (on a C57BL/JN background), aged 4 months old and weighing 40 to 45 g, and wild-type (WT) C57BL/JN mice, weighing 20-22 g, were purchased from the Model Animal Research Center of Nanjing University in China. The mice were housed under environmentally controlled conditions with a 12-h light-dark cycle and a standard diet and water. All animals experimental procedures were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University.

Permanent middle cerebral artery occlusion (pMCAO)

The mice were anesthetized with intraperitoneal injection of sodium pentobarbital (75 mg/kg) (Sigma). A skin incision was made between the right eye and the right ear, and the temporal muscle was removed to perform the craniotomy. After resecting the dura mater, the distal portion of the middle cerebral artery (MCA) was exposed and permanently electrocoagulated. The sham operation only exposed the MCA, but the electrocoagulation was not performed. Acute stress hyperglycemia was induced with intraperitoneal injection of 0.2 ml 50% glucose immediately after electrocoagulation, followed by repeated injections every four hours until sacrifice 24 hours later. Mice were intraperitoneally injected with CI-Amidine (Cayman Chemical, Michigan) half an hour before electrocoagulation of the MCA. Blood glucose was

detected in the tail vein using the analyzer (Performa, Basel, Switzerland) before the operation, immediately after the MCA electrocoagulation and at the indicated times after the operation.

Measurement of infarction volume

The mice were deeply anesthetized and sacrificed at the indicated time points. The brains were rapidly frozen at -20°C for 10 min and then sectioned into 2 mm coronal slices and stained with 2,3,5-triphenyltetrazolium chloride. Images were taken, and the infarction area was calculated by using ImageJ (version 1.47t; National Institutes of Health, Bethesda, MD; <http://imagej.nih.gov/ij/>). The percentage of infarction volume was calculated by the following equation: Infarction volume (%) = Infarction volume/Total brain volume*100%.

Immunofluorescence and immunohistochemical staining

The mice were perfused transcardially with pre-cooled 4% paraformaldehyde (pH 7.4; Sigma) under deep anesthesia. The brains of the mice and the thrombi of stroke patients were embedded in paraffin and cut into 5 µm sections. For immunofluorescence staining, the sections were immersed in 0.2% Triton X-100 for permeabilization and in 10% goat serum for blocking and were then incubated with primary antibodies against Ly6G (1:400, BD Biosciences) and H3Cit (1:400, Abcam) overnight at 4°C and subsequently two secondary antibodies (anti-rat IgG coupled to FITC 488, 1:1000; anti-rabbit IgG coupled to DyLight 594, 1:1000 (Jackson Immuno Research)) for 1 hour at room temperature. Nuclei were stained with DAPI. The slices were then mounted with coverslips using antifade reagent (Molecular Probes, Invitrogen) and sealed with nail polish. Images were acquired with a laser confocal microscope (SP8; Leica Microsystems, Wetzlar, Germany). For immunohistochemical staining, the sections were rinsed and the endogenous peroxidase activity was blocked with 3% H₂O₂. Then, the sections were blocked with 5% goat serum and incubated with diluted primary antibodies against H3Cit (1:200, Abcam) overnight at 4°C. A biotinylated secondary anti-rabbit IgG antibody (1:1000, Roche) was added for 30 min, followed by the addition of Sav-HRP conjugates and incubation at room temperature for another 30 min. Diaminobenzidine (DAB) was applied to reveal the color of antibody staining. The stained slides were imaged using a light microscope (SP8; Leica Microsystems) and CaseViewer software. ImageJ software was used to analyze the H3Cit content. Five 20× fields of view per section stained for H3Cit were chosen at random and used to calculate the average amount of H3Cit in each thrombus. Classical H&E staining was conducted based on a routine procedure.

Western blotting

Peri-infarction brain tissues were lysed in the ice-cold radio immunoprecipitation assay buffer (Thermo Fisher Scientific) with protease and phosphatase inhibitors. Protein extracts were separated by SDS-PAGE. The proteins were transferred to PVDF membranes. After blocking with 5% skimmed milk, the membranes were incubated with the diluted primary antibodies against H3Cit (Abcam). GAPDH was used as the internal standard. After washing with TBST buffer, the membranes were incubated with secondary antibodies (IRDye-700 nm anti rabbit and IRDye-800 nm anti mouse) (Roche) and photographed using an

Odyssey imaging system (LI-COR Bioscience). The measurement of band immunofluorescence intensity was determined by Quantity One (Bio-Rad).

Enzyme-linked immunosorbent assay

Inflammatory cytokines in serum were evaluated by using an ELISA kit (Mouse TNF alpha uncoated ELISA, 88-7324; Mouse IL-1 β uncoated ELISA, 88-7013; Mouse IL-6 uncoated ELISA, 88-7064; Thermo Fisher Scientific) according to the manufacturer's instructions. Each assay was performed in triplicate.

Evaluation of neurological deficits

A rotarod test was performed (Columbus Instruments, America) for four consecutive days before pMCAO for learning and at 24 hours after pMCAO. The measurement of the last day before pMCAO was defined as the preoperation test. A grip strength test (Bio-ACTIVW-M, Bioseb, France) was used to evaluate the motor function on the day before and at 24 hours after pMCAO.

Statistical analysis

The results are presented as the mean $\pm$ standard deviation (SD). Data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA) and SigmaStat 4.0 (Systat Software). Normality was tested using the Kolmogorov–Smirnov test. Kruskal-Wallis with Dunn's method was used to assess H3Cit-positive areas between different groups. Repeated measures ANOVA and post hoc Dunnett's and Bonferroni's multiple comparison tests were used to confirm significant differences. A $p < 0.05$ was considered significant.

Results

NETs were induced by diabetes and hyperglycemia in the thrombi of stroke patients

We collected thrombi from 41 ischemic stroke patients after the thrombectomy procedure, of which 9 were confirmed as having diabetes, 7 were confirmed as having acute hyperglycemic non-diabetes and 25 were normoglycemic patients. H&E staining showed an abundance of granulocytes in the thrombi (Figure 1A, B, C). Nuclear material that appeared to be extracellular was found in the magnified H&E pictures (Figure 1D, E, F). Staining for H3Cit, a defining marker of NETs, confirmed the formation of NETs in these thrombi (Figure 1G, H, I). The content of H3Cit was greatly increased in the area that had abundant granulocytes, as shown in representative images from the three different groups of patients (Figure 1J, K, L). Quantitative analysis showed that H3Cit content was relatively low in the normoglycemic stroke patients ($0.25\% \pm 0.19\%$), while it was more variable and higher in diabetic ($1.10\% \pm 1.0\%$, $p < 0.05$) and acute hyperglycemic patients ($0.81\% \pm 0.48\%$, $p < 0.05$) (Figure 1M).

To further confirm the presence of NETs in the thrombi, immunofluorescent staining was performed combining H3Cit with DNA dye (DAPI). From the representative patient images, we clearly showed that H3Cit accompanied DNA that was released from the nucleus (Figure 2).

Blood glucose measurements of the pMCAO mice

Blood glucose measurements from the tail vein are shown in figure 3. Animals had free access to food and water. The basal level of blood glucose in the db/db mice exceeded the upper limit (35 mmol/L) of the analyzer and was 9.68 ± 0.54 mmol/L in WT mice before the operation. Immediately after the operation, there was a transient but significant increase in blood glucose in the WT (20.10 ± 3.57 mmol/L) and WT+HG mice (18.62 ± 1.80 mmol/L). Two hours after the operation, significantly higher levels of blood glucose in WT+HG mice were maintained until the sacrifice compared to those of the WT mice ($p < 0.001$). Although the blood glucose in db/db mice decreased spontaneously, the levels of blood glucose were much higher in the db/db mice than in the WT mice until 20 hours after the operation ($p < 0.05$) (Figure 3).

Neutrophils and NETs were increased in diabetic and acute hyperglycemic pMCAO mice

To detect neutrophil infiltration in ischemic brain tissue, the peri-infarction brain tissue was immunofluorescently stained for the neutrophil marker Ly6G. Compared to the levels in WT mice at 24 h after MCAO, the number of infiltrated neutrophils was much higher in the brains of db/db and WT+HG mice (Figure 4A). Next, we evaluated H3Cit in the peri-infarction brain tissue. In the normoglycemic WT MCAO mice, some star dot signals of H3Cit appeared intracellularly, indicative of reduced and early NETosis. These results were completely different from the strong H3Cit string signals that appeared extracellularly in db/db MCAO and WT+HG MCAO mice, which are indicative of increased and complete NETosis (Figure 4B). To further confirm the NETs in peri-infarction brain tissue, we used the PAD4 inhibitor Cl-Amidine to inhibit NETosis in MCAO mice. In db/db mice, compared to expression in the sham MCAO mice, H3Cit expression increased significantly (0.57 ± 0.11 versus 0.29 ± 0.08 , $p < 0.05$), which was inhibited when Cl-Amidine was administered (0.36 ± 0.08 versus 0.57 ± 0.11 , $p < 0.05$) (Figure 4C). In the WT MCAO mice with normoglycemia, there was a significant increase in H3Cit protein expression compared to that of the sham MCAO mice (0.25 ± 0.10 versus 0.09 ± 0.01 , $p < 0.05$). Moreover, the WT MCAO mice with hyperglycemia contained double the amount of H3Cit (0.54 ± 0.12) compared to that of the WT MCAO mice with normoglycemia (0.25 ± 0.10 , $p < 0.05$) (Figure 4D). H3Cit expression was inhibited after injection of Cl-Amidine in both normoglycemic and hyperglycemic MCAO mice.

Inhibition of NETosis decreased the infarction in diabetic and hyperglycemic stroke animals

In db/db MCAO mice, the infarction volume was much larger than in the WT MCAO mice ($30.64\% \pm 3.62\%$ versus $22.34\% \pm 2.39\%$, $p < 0.01$). This difference in infarction volume between db/db MCAO mice and WT MCAO mice disappeared when Cl-amidine was injected intraperitoneally at a low dose of 30 mg/kg or a high dose of 60 mg/kg before the operation. Compared to the infarction volume in the db/db MCAO mice injected with vehicle, Cl-amidine at a dose of 60 mg/kg significantly decreased the infarction volume ($30.64\% \pm 3.62\%$ versus $21.76\% \pm 3.61\%$, $p < 0.05$) (Figure 5A). In WT MCAO mice, the increase in infarction volume was also shown in the WT mice with hyperglycemia induced by glucose injection compared to that of the WT mice with normoglycemia ($36.38\% \pm 3.77\%$ versus $22.34\% \pm 2.39\%$, $p < 0.01$). This difference in infarction volume between WT MCAO mice and WT+HG MCAO mice diminished but remained

significant when Cl-amidine was injected intraperitoneally. As in the db/db mice, the high dose of Cl-amidine also decreased the infarction volume in WT MCAO mice with hyperglycemia compared to that of the WT MCAO mice with normoglycemia ($36.38\pm 3.77\%$ versus $29.32\pm 3.13\%$, $p<0.05$) (Figure 5B).

Inhibition of NETosis alleviated neurological deficits in diabetic and hyperglycemic stroke animals

Forelimb grip strength and rotarod tests were performed to evaluate neurological function in the pMCAO animal model. The results of the pre-operative tests were defined as the baseline neurological function. In db/db mice, there were no significant differences between sham operation and pMCAO mice as shown by forelimb grip strength and rotarod tests before operation. An obvious decrease in both forelimb grip strength and rotarod time was found in pMCAO mice compared to those of the sham group (36.59 ± 12.2 g versus 74.28 ± 3.85 g, 48.07 ± 10 s versus 113.01 ± 8.9 s, $p<0.05$). When compared to the grip strength of db/db pMCAO mice, Cl-amidine reversed the effect on grip strength (60.42 ± 10.57 g versus 36.59 ± 12.2 g, $p<0.05$) (Figure 6A). In the rotarod test, compared to the time of the sham db/db mice, the rotarod time was much shorter in db/db pMCAO mice (48.07 ± 10.0 s versus 113.01 ± 8.90 s, $p<0.05$). The rotarod time was significantly increased in db/db pMCAO mice injected with Cl-amidine (78.13 ± 9.2 s, $p<0.05$) (Figure 6C). In WT mice, no significant differences in grip strength and rotarod test were observed pre-operation. Compared to the grip strength of normoglycemic pMCAO WT mice, there was a large grip strength loss in hyperglycemic pMCAO WT mice (66.57 ± 14.53 g versus 98.7 ± 10.95 g, $p<0.05$). However, Cl-amidine reversed the loss of grip strength significantly (90.17 ± 13.21 g versus 66.57 ± 14.53 g, $p<0.05$) (Figure 6B). In the rotarod test, all pMCAO WT mice showed obvious deficits. This is more significant in hyperglycemic pMCAO WT mice compared to that of the normoglycemic pMCAO WT mice (36.81 ± 14.53 s versus 68.78 ± 10.95 s, $p<0.01$). When compared to the time of the hyperglycemic pMCAO WT mice, Cl-amidine restored the rotarod time (60.1 ± 13.21 s versus 36.81 ± 14.53 s, $p<0.05$) (Figure 6D).

Inflammation associated with NETosis in diabetic and hyperglycemic stroke animals

Inflammatory factors associated with NETosis were evaluated in diabetic and hyperglycemic stroke animals. A two-fold increase in IL-1 β was detected in the serum of pMCAO db/db mice compared to that of the sham group (102.26 ± 20 pg/ml versus 29.4 ± 5.6 pg/ml, $p<0.001$). There was a restoration in the pMCAO plus Cl-amidine group (64.96 ± 10.9 pg/ml, $p<0.05$) (Figure 7A). TNF- α in the serum of pMCAO db/db mice was doubled when compared to that of the sham group (8.24 ± 0.8 pg/ml versus 3.37 ± 0.5 pg/ml, $p<0.001$). This phenomenon was diminished in pMCAO db/db mice treated with Cl-amidine (5.68 ± 1.0 pg/ml versus 8.24 ± 0.8 pg/ml, $p<0.05$) (Figure 7C). In WT mice, both TNF- α (73.01 ± 10.10 pg/ml versus 12.59 ± 2.87 pg/ml, $p<0.001$) and IL-1 β (274.68 ± 15.13 pg/ml versus 195.6 ± 20.12 pg/ml, $p<0.001$) increased in hyperglycemic pMCAO mice compared to those of the normoglycemic pMCAO mice (Figure 7B). Moreover, both TNF- α (22.0 ± 5.10 pg/ml versus 73.01 ± 10.10 pg/ml, $p<0.05$) and IL-1 β (171.33 ± 10.14 pg/ml versus 274.68 ± 15.13 pg/ml, $p<0.05$) in hyperglycemic pMCAO WT mice injected with Cl-amidine decreased significantly (Figure 7D). No significant change in IL-6 content was observed in db/db or WT mice with or without Cl-amidine injection (Figure 7E, F).

Discussion

In this study, we showed that (1) hyperglycemia primed the formation of NETs in thrombi of stroke patients and peri-infarction brain tissue of pMCAO mice; (2) NETs play a role in thrombosis in diabetic ischemic stroke; (3) NETs exacerbate ischemic brain damage in diabetes; and (4) the inhibition of NETosis could eliminate the harmful effect of hyperglycemia on ischemic brain injury.

Thrombus constitution provides hints on thrombus formation. Therefore, we analyzed the composition of thrombi in stroke patients. Previous studies showed that NETs contributed to coagulation and platelet aggregation, thus promoting thrombosis[19], such as deep vein thrombosis[20] and thrombi in acute myocardial infarction[21]. Although the NETs were verified in thrombi retrieved from large vessel occlusion stroke patients and were associated with a cardioembolic origin, the broad range of NET amounts among the different thrombi suggested that other factors may affect NETosis[18]. Early reports showed elevated levels of circulating NETs in type 2 diabetes[15]. Here, we confirmed an abundance of NET formation in thrombi of stroke patients that was associated with high blood glucose, either in diabetes or acute stress hyperglycemia. These results indicate that hyperglycemia primes NET formation in the thrombi of stroke patients. NETs play an important role in thrombosis in ischemic stroke.

Neutrophil granulocytes have been key players in cerebral ischemia[22]. The release of proteases associated with decondensed DNA triggers neurotoxicity[23]. Histones are released into the extracellular space, activating the immune system and causing further cytotoxicity[24, 25]. Therefore, we hypothesize that NET formation accelerates and exacerbates injury, especially in hyperglycemia. Hyperglycemia upon admission is attributed to existing chronic diabetes and acute stressful hyperglycemia[26]. To simulate the clinical scenario, we used db/db mice, WT mice and WT mice injected with glucose to establish pMCAO models for simulating the three kinds of ischemic stroke patients based on blood glucose. The results showed that induction of hyperglycemia was successful in db/db and WT+HG mice after pMCAO. There was a significant but transient rise in blood glucose immediately after the operation, which may be attributed to the stress of the operation. Blood glucose dropped gradually in WT mice and more obviously in db/db mice after the operation. Loss of appetite after the operation may explain this effect. In the pMCAO mice, we verified that NETs increased in ischemic brain tissue, which is in line with a recent report[27]. Moreover, the NET content in ischemic brain tissue was more highly induced in hyperglycemia. Then, as expected, we found that infarct volume was much larger in db/db and hyperglycemic pMCAO mice compared to those of the normoglycemic mice. This may be explained by the contribution of NETs to thrombus stabilization and growth[21] or the direct cytotoxicity of histones[24].

Since neutrophils and NETs orchestrate the initiation and modulation of inflammation[28, 29], we evaluated the proinflammatory cytokines produced in pMCAO mice. TNF- α and IL-1 β increased in db/db and hyperglycemic mice, which indicates a proinflammatory role of hyperglycemia. The downregulation of TNF- α and IL-1 β after NET inhibition demonstrates that NETs drive inflammatory responses in pMCAO mice, as shown in other reports[30, 31].

In this study, we employed a pMCAO model other than the tMCAO mouse model. The infarction area was more uniform in individuals with pMCAO than in those with tMCAO. Although the most effective treatment for ischemic stroke is rapid recanalization, the proportion of patients who receive thrombolysis or mechanical thrombectomy is still very low. Most patients suffer permanent ischemia. Neutrophils could be transported to the brain by reperfusion and produce more NETs in tMCAO. This is not contradictory to the results of the present study.

Conclusions

In conclusion, our results showed that hyperglycemia activates neutrophils to overproduce NETs and identified NETs as a key factor that exacerbates ischemic brain damage in diabetes. The upstream signaling pathways controlling NETosis in hyperglycemia or diabetes need further exploration.

Abbreviations

NETs: Neutrophil extracellular traps; H3Cit: citrullinated histone H3; pMCAO: permanent middle cerebral artery occlusion; rtPA: recombinant human tissue plasminogen activator; cfDNA: cell-free DNA; NG: normoglycemia; DM: diabetes mellitus; HG: acute hyperglycemia; WT: wild-type; MCA: middle cerebral artery.

Declarations

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable

Acknowledgments

We appreciate that professor Biaoqiao Zhao help us design the animal study.

Funding

This research was funded by Shanghai Municipal Commission of Health and Family Planning(201840099)

Availability of data and materials

All data generated or analyzed during this work are included in this article.

Authors' contributions

JSD, FZ and YWZ designed the research. JSD collected the clinical data. FZ and YLZ performed the animal experiments. JSD contributed to the production of the manuscript. YJZ, XFX and FW performed the data analysis. XJZ revised the manuscript. All authors read and approved the final manuscript.

Ethics approval

All the animal experiments were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University.

References

- 1.Uyttenboogaart M, Koch MW, Stewart RE, Vroomen PC, Luijckx GJ, De Keyser J: *Moderate hyperglycaemia is associated with favourable outcome in acute lacunar stroke. Brain* 2007, *130*:1626–1630.
- 2.Capes SE, Hunt D, Malmberg K, Pathak P, Gerstein HC: *Stress hyperglycemia and prognosis of stroke in nondiabetic and diabetic patients: a systematic overview. Stroke* 2001, *32*:2426–2432.
- 3.Shimoyama T, Kimura K, Uemura J, Saji N, Shibazaki K: *Elevated glucose level adversely affects infarct volume growth and neurological deterioration in non-diabetic stroke patients, but not diabetic stroke patients. Eur J Neurol* 2014, *21*:402–410.
- 4.Kim JT, Jahan R, Saver JL: *Impact of Glucose on Outcomes in Patients Treated With Mechanical Thrombectomy: A Post Hoc Analysis of the Solitaire Flow Restoration With the Intention for Thrombectomy Study. Stroke* 2016, *47*:120–127.
- 5.Bruno A, Levine SR, Frankel MR, Brott TG, Lin Y, Tilley BC, Lyden PD, Broderick JP, Kwiatkowski TG, Fineberg SE: *Admission glucose level and clinical outcomes in the NINDS rt-PA Stroke Trial. Neurology* 2002, *59*:669–674.
- 6.Erdur H, Polymeris A, Grittner U, Scheitz JF, Tutuncu S, Seiffge DJ, Audebert HJ, Nolte CH, Engelter ST, Rocco A: *A Score for Risk of Thrombolysis-Associated Hemorrhage Including Pretreatment with Statins. Front Neurol* 2018, *9*:74.
- 7.Demchuk AM, Tanne D, Hill MD, Kasner SE, Hanson S, Grond M, Levine SR: *Predictors of good outcome after intravenous tPA for acute ischemic stroke. Neurology* 2001, *57*:474–480.
- 8.Baird TA, Parsons MW, Phan T, Butcher KS, Desmond PM, Tress BM, Colman PG, Chambers BR, Davis SM: *Persistent poststroke hyperglycemia is independently associated with infarct expansion and worse clinical outcome. Stroke* 2003, *34*:2208–2214.
- 9.Els T, Klisch J, Orszagh M, Hetzel A, Schulte-Monting J, Schumacher M, Lucking CH: *Hyperglycemia in patients with focal cerebral ischemia after intravenous thrombolysis: influence on clinical outcome and infarct size. Cerebrovasc Dis* 2002, *13*:89–94.

10. Johnston KC, Bruno A, Pauls Q, Hall CE, Barrett KM, Barsan W, Fansler A, Van de Bruinhorst K, Janis S, Durkalski-Mauldin VL, et al: *Intensive vs Standard Treatment of Hyperglycemia and Functional Outcome in Patients With Acute Ischemic Stroke: The SHINE Randomized Clinical Trial*. *JAMA* 2019, 322:326–335.
11. Jorch SK, Kubes P: *An emerging role for neutrophil extracellular traps in noninfectious disease*. *Nat Med* 2017, 23:279–287.
12. Doring Y, Soehnlein O, Weber C: *Neutrophil Extracellular Traps in Atherosclerosis and Atherothrombosis*. *Circ Res* 2017, 120:736–743.
13. Menegazzo L, Ciciliot S, Poncina N, Mazzucato M, Persano M, Bonora B, Albiero M, Vigili de Kreutzenberg S, Avogaro A, Fadini GP: *NETosis is induced by high glucose and associated with type 2 diabetes*. *Acta Diabetol* 2015, 52:497–503.
14. Rodriguez-Espinosa O, Rojas-Espinosa O, Moreno-Altamirano MM, Lopez-Villegas EO, Sanchez-Garcia FJ: *Metabolic requirements for neutrophil extracellular traps formation*. *Immunology* 2015, 145:213–224.
15. Miyoshi A, Yamada M, Shida H, Nakazawa D, Kusunoki Y, Nakamura A, Miyoshi H, Tomaru U, Atsumi T, Ishizu A: *Circulating Neutrophil Extracellular Trap Levels in Well-Controlled Type 2 Diabetes and Pathway Involved in Their Formation Induced by High-Dose Glucose*. *Pathobiology* 2016, 83:243–251.
16. Wong SL, Demers M, Martinod K, Gallant M, Wang Y, Goldfine AB, Kahn CR, Wagner DD: *Diabetes primes neutrophils to undergo NETosis, which impairs wound healing*. *Nat Med* 2015, 21:815–819.
17. Valles J, Lago A, Santos MT, Latorre AM, Tembl JI, Salom JB, Nieves C, Moscardo A: *Neutrophil extracellular traps are increased in patients with acute ischemic stroke: prognostic significance*. *Thromb Haemost* 2017, 117:1919–1929.
18. Laridan E, Denorme F, Desender L, Francois O, Andersson T, Deckmyn H, Vanhoorelbeke K, De Meyer SF: *Neutrophil extracellular traps in ischemic stroke thrombi*. *Ann Neurol* 2017, 82:223–232.
19. Martinod K, Wagner DD: *Thrombosis: tangled up in NETs*. *Blood* 2014, 123:2768–2776.
20. Brill A, Fuchs TA, Savchenko AS, Thomas GM, Martinod K, De Meyer SF, Bhandari AA, Wagner DD: *Neutrophil extracellular traps promote deep vein thrombosis in mice*. *J Thromb Haemost* 2012, 10:136–144.
21. de Boer OJ, Li X, Teeling P, Mackaay C, Ploegmakers HJ, van der Loos CM, Daemen MJ, de Winter RJ, van der Wal AC: *Neutrophils, neutrophil extracellular traps and interleukin-17 associate with the organisation of thrombi in acute myocardial infarction*. *Thromb Haemost* 2013, 109:290–297.
22. Strecker JK, Schmidt A, Schabitz WR, Minnerup J: *Neutrophil granulocytes in cerebral ischemia - Evolution from killers to key players*. *Neurochem Int* 2017, 107:117–126.

- 23.Allen C, Thornton P, Denes A, McColl BW, Pierozynski A, Monestier M, Pinteaux E, Rothwell NJ, Allan SM: *Neutrophil cerebrovascular transmigration triggers rapid neurotoxicity through release of proteases associated with decondensed DNA. J Immunol* 2012, *189*:381–392.
- 24.Silk E, Zhao H, Weng H, Ma D: *The role of extracellular histone in organ injury. Cell Death Dis* 2017, *8*:e2812.
- 25.Gilthorpe JD, Oozeer F, Nash J, Calvo M, Bennett DL, Lumsden A, Pini A: *Extracellular histone H1 is neurotoxic and drives a pro-inflammatory response in microglia. F1000Res* 2013, *2*:148.
- 26.Luitse MJ, Biessels GJ, Rutten GE, Kappelle LJ: *Diabetes, hyperglycaemia, and acute ischaemic stroke. Lancet Neurol* 2012, *11*:261–271.
- 27.Kim SW, Lee H, Lee HK, Kim ID, Lee JK: *Neutrophil extracellular trap induced by HMGB1 exacerbates damages in the ischemic brain. Acta Neuropathol Commun* 2019, *7*:94.
- 28.Hahn J, Knopf J, Maueroeder C, Kienhofer D, Leppkes M, Herrmann M: *Neutrophils and neutrophil extracellular traps orchestrate initiation and resolution of inflammation. Clin Exp Rheumatol* 2016, *34*:6–8.
- 29.Castanheira FVS, Kubes P: *Neutrophils and NETs in modulating acute and chronic inflammation. Blood* 2019, *133*:2178–2185.
- 30.Song C, Li H, Li Y, Dai M, Zhang L, Liu S, Tan H, Deng P, Liu J, Mao Z, et al: *NETs promote ALI/ARDS inflammation by regulating alveolar macrophage polarization. Exp Cell Res* 2019, *382*:111486.
- 31.Shao S, Fang H, Dang E, Xue K, Zhang J, Li B, Qiao H, Cao T, Zhuang Y, Shen S, et al: *Neutrophil Extracellular Traps Promote Inflammatory Responses in Psoriasis via Activating Epidermal TLR4/IL–36R Crosstalk. Front Immunol* 2019, *10*:746.

Figures

patient blood glucose (M) (NG: normoglycemia, DM: diabetes mellitus, HG: hyperglycemia). * $p < 0.05$.
Scale bars: 50 μm .

Figure 2

Immunofluorescent identification of NETs in stroke thrombi. Confocal images clearly showed that H3Cit accompanied DNA release from the nucleus. Scale bar: 50 μm .

Figure 3

Blood glucose measurements of the pMCAO mice. Compared to the WT group, *** $p < 0.001$, ### $p < 0.001$, * $p < 0.05$. $n = 5$.

Figure 4

Infiltration of neutrophils and increased NETs in pMCAO mice was exacerbated by diabetes and hyperglycemia. The neutrophil marker Ly6G was stained in the peri-infarct brain tissue. More neutrophils appeared in db/db and WT+HG mice (A). H3Cit, the NET marker, was stained in the peri-infarct brain tissue. Typical H3Cit released extracellularly formed NETs in db/db and WT+HG mice (B). Western blot detection of H3Cit in peri-infarct brain tissue of db/db pMCAO mice (C) and WT and WT+HG mice (D). In normoglycemic WT mice, there was a moderate increase in H3Cit content after pMCAO. In diabetic and WT+HG mice, a large increase in H3Cit content was observed after pMCAO ($n = 5$). WT: wild type. HG:

hyperglycemia. CI: CI-Amidine. * $p < 0.05$, compared to the sham group. # $p < 0.05$, ## $p < 0.01$, compared to the pMCAO group. & $p < 0.001$, compared to the PMCAO+HG group. Scale bars: 50 μm .

Figure 5

Inhibition of NETosis decreased the infarction in diabetic and hyperglycemic stroke animals. 2,3,5-Triphenyltetrazolium chloride staining of mouse brain infarction tissue. Larger infarction volume in db/db compared to WT mice (A) and in WT+HG compared to WT mice (B). CI-Amidine reduced the volume of infarction in both db/db and WT+HG mice ($n=5$). CI-30, CI-Amidine 30 mg/kg. CI-60, CI-Amidine 60 mg/kg. ** $p < 0.01$, compared to the WT mice administered vehicle. # $p < 0.05$, compared to the db/db mice administered vehicle. & $p < 0.05$, compared to the WT+HG mice administered vehicle.

Figure 6

Inhibition of NETosis alleviated neurological deficits in diabetic and hyperglycemic stroke animals. Grip strength (A, B) and Rotarod test (C, D) were used to evaluate the neurological deficit after pMCAO in db/db (A, C) and WT mice (B, D). CI-Amidine reduced the loss of grip strength in db/db (A) and WT+HG mice (B) and rotarod time in both db/db (C) and WT+HG mice (D) (n=8). *p<0.05, compared to the Sham group. #p<0.05, compared to the pMCAO group. &p<0.05, compared to the pMCAO+HG group.

Figure 7

Inflammation associated with NETosis in diabetic and hyperglycemic stroke animals. The levels of IL-1 β (A, B), TNF- α (C, D) and IL-6 (E, F) were evaluated after pMCAO in db/db (A, C, E) and WT mice (B, D, F) by ELISA. IL-1 β (A) and TNF- α (C) increased in db/db mice compared to those of the sham mice. IL-1 β (B) and TNF- α (D) increased in WT+HG mice compared to those of the WT pMCAO mice. CI-Amidine reduced the expression of IL-1 β (A, B) and TNF- α (C, D). No obvious change in IL-6 was observed in any of the groups (n=6). ***p<0.001, compared to the sham pMCAO group. #p<0.05, ###p<0.001, compared to the pMCAO group. &p<0.01, compared to the pMCAO+HG group.