Dietary Intake of Sugar Substitutes Aggravates Cerebral Ischemic Injury and Impairs Endothelial Progenitor Cells in Mice

Xiao-Hui Dong, MSc; Xin Sun, MSc; Guo-Jun Jiang, PhD; Alex F. Chen, PhD; He-Hui Xie, PhD

Background and Purpose—In our current food supply, sugar substitutes are widely used in beverages and other food products. However, there is limited information about the link between dietary consumption of sugar substitutes and stroke to date. This study sought to determine the effect of various sugar substitutes on the cerebral ischemic injury and endothelial progenitor cells, which have been implicated to play an important role in vascular repair and revascularization in ischemic brain tissues, in mice.

Methods—After treatment with sucrose and various sugar substitutes (the doses are in the range of corresponding acceptable daily intake levels) and vehicle for 6 weeks, mice were subjected to permanent left middle cerebral artery occlusion, and the infarct volumes, angiogenesis, and neurobehavioral outcomes were determined. In addition, the number and function of endothelial progenitor cells were also examined.

Results—After long-term treatment with fructose, erythritol (sugar alcohols), acesulfame K (artificial sweeteners), or rebaudioside A (rare sugars), the cerebral ischemic injury (both infarct volumes and neurobehavioral outcomes) was significantly aggravated, angiogenesis in ischemic brain was reduced, and endothelial progenitor cell function was impaired in mice compared with control. However, the similar impairments were not found in sucrose (with the same dose as fructose’s)-treated mice.

Conclusions—Long-term consumption of sugar substitutes aggravated cerebral ischemic injury in mice, which might be partly attributed to the impairment of endothelial progenitor cells and the reduction of angiogenesis in ischemic brain. This result implies that dietary intake of sugar substitutes warrants further attention in daily life.

Key Words: endothelial progenitor cells ■ sweetening agents

As the negative impact of sugar consumption on weight and other health outcomes has been increasingly recognized, many persons have turned to fructose and sugar substitutes (like artificial sweeteners, sugar alcohols, and rare sugars) as a way to reduce the risk of these consequences. But recently, accumulating evidences suggest that dietary consumption of these compounds may also increase the risk of metabolic disorder, which is one of major risk factors for cerebrocardiovascular diseases, such as stroke. Stroke is a devastating disease and the major cause of mortality and morbidity worldwide. However, there is limited information about the link between dietary consumption of sugar substitutes and stroke to date.

The integrity and function of the endothelium plays an important role in the prevention of cerebrocardiovascular diseases, such as stroke. Endothelial progenitor cells (EPCs) are immature cells that can differentiate into mature endothelial cells and could be recruited from bone marrow to the injury site to promote endothelial regeneration and neovascularization. In addition, EPCs have been used to successfully improve function recovery in ischemic organs, including brain after ischemic injury. Recent reports showed that EPCs may serve as a new marker for stroke outcomes. Therefore, EPC dysfunction and the consequent abnormality of endothelial regeneration may influence the susceptibility to cerebral ischemic injury.

On the basis of these findings, this study sought to determine the effect of various sugar substitutes consumption on the cerebral ischemic injury and EPCs in mice. Among sugar substitutes widely used in beverages and other food products, fructose,
erythritol (sugar alcohols), acesulfame K (artificial sweeteners), and rebaudioside A (rare sugars) were used in this study.

Methods
Male C57BL/6 mice (10–12 week, 20–25g) used in these experiments were obtained from Sino-British SIPPR/BK Laboratory Animal Ltd (Shanghai, China). Mice were given fructose (Fru, 1.5 g/kg body weight), erythritol (Ery, 15 mg/kg body weight), acesulfame K (Ace, 4 mg/kg body weight), rebaudioside A (Reb, 1 mg/kg body weight), sucrose (Suc, 1.5 g/kg body weight), and water (Control) via drinking water for 6 weeks. The doses of these sugar substitutes (Sigma) are in the range of acceptable daily intake or estimated daily intake levels for food additives approved by US Food and Drug Administration (FDA) or European Union. Sucrose (Sigma), with the same dose as fructose’s, was denoted as positive control. The body weight was monitored every week, and fasting blood glucose levels were measured before and after 6 weeks of treatment. After long-term treatment, mice were subjected to permanent focal cerebral ischemia, and the infarct volumes, angiogenesis, and neurobehavioral outcomes were determined according to published protocols and our previous work. According to our described technique, mouse circulating EPCs and bone marrow–derived EPCs were isolated and cultured, and the functions (migration, tube formation and adhesion assay) of bone marrow–derived EPCs were examined. All animals received humane care, and the experimental procedures were in compliance with the institutional animal care guidelines.

Figure 1. Sugar substitutes aggravate cerebral ischemic injury and reduced angiogenesis in ischemic brain in mice. A, The representative images of 2,3,5-triphenyltetrazolium chloride (TTC)-stained brain sections (up) and cerebral infarct volumes (below). **P<0.01 vs control (Con), n=10 to 18. B and C, Neurobehavioral outcomes (B, Beam Test; C, Body Asymmetry Test). *P<0.05, **P<0.01 vs Con; n=10 to 21. D, The local angiogenesis in the ischemic brain in mice. CD31 immunostaining shows microvessels in ischemic brain of mice treated with sugar substitutes. The bar graph shows that the number of microvessels in sugar substitutes–treated mice was significantly reduced compared with that in vehicle-treated mice. **P<0.01 vs Con, n=6. Scale bar= 50 μm (left); 25 μm (right). Ace indicates acesulfame K; Ery, erythritol; Fru, fructose; Reb, rebaudioside A, and Suc, sucrose.
Detailed methods are described in the online-only Data Supplement.

Statistical Analysis
Data are expressed as mean±SEM. Statistical significance of difference among groups was performed using 1-way ANOVA followed by Tukey post hoc analysis. A value of \( P < 0.05 \) was considered statistically significant.

Results
Effects of Sugar Substitutes on Fasting Blood Glucose and Body Weight in Mice
There was no significant difference in baseline fasting blood glucose levels among all the groups of mice (data not shown). After 6 weeks of treatment, compared with control mice, fasting blood glucose levels were slightly but significantly increased in acesulfame K–treated mice but not in mice treated with fructose, erythritol, rebaudioside A, or sucrose (Figure 1A in the online-only Data Supplement). There was no significant difference in body weights between sugar substitutes–treated mice and control animals (Figure 1B in the online-only Data Supplement).

Sugar Substitutes Aggravate Cerebral Ischemic Injury and Reduced Angiogenesis in Ischemic Brain in Mice
The infarct volumes were significantly increased and the corresponding neurobehavioral outcomes were markedly impaired in sugar substitutes–treated, but not sucrose (with the same dose as fructose’s)-treated, mice compared with control (Figure 1A–1C). Furthermore, angiogenesis in ischemic brain was assessed at 3 days after cerebral ischemia. It

Figure 2. Effects of sucrose and sugar substitutes on the number of circulating endothelial progenitor cells (EPCs) and functions of bone marrow–derived EPCs (BM-EPCs) in mice. A, The number of circulating EPCs determined by Sca-1/Flk-1 double-staining flow cytometry. n=7 to 17. B, Migration assay of BM-EPCs. C, Tube formation assay of BM-EPCs. D, Adhesion assay of BM-EPCs. n=5 to 12, \( *P < 0.01 \) vs control (Con). Ace indicates acesulfame K; Ery, erythritol; Fru, fructose; Reb, rebaudioside A, hpf, high-power fields (magnification ×100); and Suc, sucrose. Scale bar=100 μm.
Chronic intake of sugar substitutes  

↓  

EPC functions  

↓  

Angiogenesis in ischemic brain  

↓  

Cerebral infarct volumes  

↑  

EPC functions  

↓  

Neurobehavioral outcomes  

Figure 3. Putative mechanisms underlying sugar substitutes aggravating cerebral ischemic injury in mice. Chronic sugar substitutes intake impaired endothelial progenitor cell (EPC) functions, reduced the angiogenesis in ischemic brain, increased cerebral infarct volumes, and decreased the corresponding neurobehavioral outcomes in mice.

was found that capillary density was significantly lower in the sugar substitutes–treated, but not sucrose-treated, mice compared with control (Figure 1D). These results suggest that long-term consumption of these sugar substitutes was able to aggravate cerebral ischemic injury and reduce angiogenesis in ischemic brain in mice.

Sugar Substitutes Impair EPCs in Mice

No significant difference in the number of circulating EPCs (Sca-1/Flk-1 double-positive cells) was found among all the groups of mice (Figure 2A). However, bone marrow–derived EPC functions (including migration, tube formation, and adhesion function) were significantly impaired in sugar substitutes–treated, but not sucrose-treated, mice compared with control (Figure 2B–2D).

Discussion

This study showed the first direct evidence that long-term intake of various sugar substitutes in the amounts currently consumed impaired EPC functions and aggravated cerebral ischemic injury in mice. However, the similar impairments were not found in sucrose (with the same dose as fructose’s)-treated mice.

Fructose in the amounts currently consumed has been proposed to be hazardous to the health of some people, but the other 3 sugar substitutes used in the present work are generally regarded as safe for consumption.1–3 It is found that neither plasma glucose nor insulin levels are affected by sugar alcohols (including erythritol) consumed.1–3 Five artificial sweeteners (including ascesulfame K) have been approved for human use by FDA and have gained popularity as a solution in maintaining the palatability of foods while substantially reducing or eliminating caloric content for many years.1–3 Rebaudioside A, a representative of rare sugars, has been regarded as safe for consumption in Asia and North America for many years and was recently permitted for use in the European Union and prized for its sweet taste and bulking properties but apparent low energy status.1–3 However surprisingly, in this study, we found that, besides fructose intake, chronic consumption of the other 3 sugar substitutes could also led to a significant increase in cerebral ischemic injury and reduction in EPC functions.

As aforementioned, EPCs participate in both vasculogenesis and vascular homestasis and have been used to successfully restore endothelial function and enhance angiogenesis in ischemic brain tissue.5–9 Therefore, EPC dysfunction and subsequent reduction of local angiogenesis in ischemic brain, as observed in this study, may partly contribute to the aggravated cerebral ischemic injury produced by chronic sugar substitutes intake (Figure 3). However, the mechanisms underlying EPC dysfunction remain to be investigated in further studies.

In addition, because EPCs has been implicated in playing an important role in vascular repair and revascularization in various ischemic organs besides brain tissue, and EPC is inversely correlated with several cardiovascular risk factors,2–5 EPC dysfunction produced by sugar substitutes might also aggravate other ischemic diseases except stroke, such as acute myocardial infarction, peripheral vascular ischemic diseases, and so on, which remains to be test by further studies.

In summary, long-term consumption of fructose, acesulfame K, rebaudioside, and erythritol might aggravate the cerebral ischemic injury, which might partly result from the impairment of EPCs and the reduction of angiogenesis in ischemic brain. This result implies that dietary intake of sugar substitutes warrants further attention in daily life. Further studies are required to better understand the mechanisms of this response.

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Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

Expanded Methods

Animal Stroke Model

After 6 weeks of treatment, mice were subjected to permanent focal cerebral ischemia according to published protocols and our previous work. 1,2 Briefly, mice were anesthetized by intraperitoneal injection of 0.1ml 3.5% chloral hydrate per 10g body weight. A skin incision was made between the ear and the orbit on the left side. After splitting of the temporalis muscle, a burr hole was drilled at the junction of the zygomatic arch and the squamous bone, through which the stem of the left middle cerebral artery (MCA) was exposed and occluded by electrocoagulation. Body temperature was maintained at 37±0.5°C by using a thermal blanket throughout the surgical procedure. Behavioral tests including Body Asymmetry Test and Beam Test were performed 3 days after MCA occlusion, and then animals were euthanized and the brains were stained with 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, USA) to determine the infarct volume as previously reported. 1,2 The regional cerebral blood flow (rCBF) was monitored by Laser-Doppler flowmetry (moorVMS-LDF1, Moor Instruments Ltd, England) before and after left middle cerebral artery occlusion. Mice with regional cerebral blood flow that was more than 15% of the baseline were excluded from the experiment. 2 All the experiments were performed in a random and blind fashion.

Behavioral tests

1) Beam Test— Beam walking across a bridge was used to assess motor coordination and balance after stroke injury. Mice were trained for 5 days before MCA occlusion to traverse a narrow round beam (5 mm diameter and 900 mm in length) to reach an enclosed escape platform. They were placed on one end of the beam and the latency to traverse the central 80% of the beam toward the enclosed escape platform at the other end was recorded. Data are expressed as mean latency to cross the beam of 3 trials. 1-3

2) Body Asymmetry Test— To measure motor asymmetry, mice were examined by elevated body swing test as described. 1-3 Mice were examined for head swings while being suspended by their tails. The direction of the swing, either right or left, was recorded when the mouse turned its head sideways by approximately at a 10° angle to the body's midline. After each swing, the mouse was allowed to move freely in a Plexiglas box for at least 30 seconds before taking the next test; the trials were repeated 20 times for each animal. The frequency of head swings toward the contralateral side was counted and normalized as follows: (number of lateral swings in 20 tests-10) /10×100%

Histological and Immunohistochemistry Assessment

After 3 days of permanent focal cerebral ischemia, the mouse was euthanized and the ischemic brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde, before being embedded in paraffin. 4 A series of 6-µm-thick sections was cut from the block. Every 10th coronal section for a total three sections was used for immunohistochemical staining. Antibody against CD31 single immunostaining was performed to detect the angiogenesis in the ischemic brain. 5

Quantification of circulating EPCs

The quantification of circulating EPCs in the peripheral blood was determined by flow cytometry according to our described technique. 6,7 Briefly, peripheral blood was obtained by cardiac puncture after mice were anesthetized. Peripheral blood mononuclear cells (PB-MNCs) were isolated by
Histopaque-1083 (Sigma) density gradient centrifugation at 400 g for 30 min. The mononuclear fraction was collected, washed in PBS (pH 7.4), red blood cells were lysed with ammonium chloride solution (Stemcell Technologies), then washed twice with PBS and once with 5% albumin bovine serum (BSA)/PBS (PBS-A). Freshly isolated PB-MNCs were re-suspended in 100 µl 15% PBS-A and incubated with antibodies to Sca-1-PE (BD Pharmingen) and Flk-1-FITC (BD Pharmingen) for 1 hour on ice. After washing and centrifugation, the cell pellets were suspended in 300 µl 2% paraformaldehyde. Quantification of Sca-1/Flk-1 double-positive cells was performed with a BD Flow cytometer (FACScan, Becton Dickenson). A non-stained sample was used to set up a threshold, and the isotype specific conjugated anti-IgG was used as a negative control. 6,7

**Bone marrow-derived EPCs (BM-EPCs) isolation and culture**

Bone marrow-derived mononuclear cells (BM-MNCs) were isolated from mouse tibia and femur as we described, 6,7 and seeded in 6-well cell culture plates coated with rat vitronectin (1 µg/mL, Sigma) at a density of 5×10⁶ cells /well, and cultured in EGM-2. After 4 days of culture, nonadherent cells were removed, and the adherent cells were further cultivated for 3 days. The adherent cells were then used for in vitro studies. 6,7

**In vitro cell function assays**

**Migration assay**

A number of 5×10⁴ cells were applied into upper Boyden’s chamber with M199. The lower chambers were loaded with M199 supplemented 50ng/ml VEGF. EPCs were allowed to migrate for 24 hours, fixed and stained Hochest 33258 (Sigma, America). The number of cells on the lower side of the membrane was counted at magnification ×100, and the mean value of 5 different areas was determined for each sample. 8

**Tube formation assay**

Matrigel-Matrix (BD Biosciences) was placed in the well of a 96-well cell culture plate and a number of 5×10⁴ EPCs were plated in each well with EGM-2. After 18 hours of incubation, images of tube morphology was taken and tube number was counted at random under 5 high-power fields (magnifications ×100) per sample. 6,7

**Adhesion assay**

In adhesion assays, 1×10⁴ cells were plated in 96-well plates coated with 1 µg/mL mouse vitronectin. After two hours of incubation, non-adherent cells were washed away and adherent cells were fixed with 2% paraformaldehyde. Nuclei were stained with Hoechst33528 (5×10⁻⁶ mol/L, 10 min, Molecular Probes). A number of adherent cells were counted at random under 5 high-power fields (magnifications ×100) per sample, and the mean value of the four wells was determined for each sample. 7

**Blood glucose level measurement**

Mice were fasted overnight and blood glucose levels were measured from tail blood with the Onetouch Ultra blood glucose monitoring system (LifeScan). 9

**References**


Supplemental Figure legends

**Supplemental Figure I.** Effect of sugar substitutes on blood glucose levels (A) and body weights (B) in mice. n=7-35. Con, Control; Fru, Fructose; Ery, Erythritol; Ace, Acesulfame k; Reb, Rebaudioside A; Suc, sucrose. *P<0.05 vs. Con.
Supplemental Figure I.

A.

![Bar chart showing blood glucose levels for different groups: Con, Fru, Ery, Ace, Reb, Suc. Bars are labeled with mean values and error bars. A significant difference is indicated with an asterisk (*).](image)

B.

![Line graph showing body weight changes over time for different groups: Con, Fru, Ery, Ace, Reb, Suc. Lines are labeled with mean values and error bars.](image)