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Insights into the authentic active ingredients and action sites of oral exogenous glutathione in the treatment of ischemic brain injury based on pharmacokinetic-pharmacodynamic studies

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Running Title Page

Active ingredients and action sites of oral exogenous GSH

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Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; BBB, blood-brain barrier; BSO, buthionine sulfoximine; CAP, captopril; CNS, central nervous system; CYS, cysteine; DAB, diaminobenzidine; DCFH-DA, 2'7 '-dichlorofluorescin diacetate; GSH, glutathione; Glu, glutamate; GLY, glycine; hCMEC/D3, human cerebral microvascular endothelial cell; H&E, hematoxylin-eosin; IL-6, interleukin-6; IL-1β, interleukin-1β; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MRM, multiple reaction monitoring; NEM, N-ethylmaleimide; OGD/R, oxygen-glucose deprivation/reperfusion; PD, Parkinson's disease; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-alpha; γ-GC, γ-Glutamylcysteine.

Abstract

Glutathione (GSH) has been reported to be closely related to various diseases of central nervous system (CNS), yet its authentic active ingredients and action sites are still unclear. In the present study, oral exogenous GSH was first proved to significantly alleviate ischemic brain injury, but it was inconsistent with its low bioavailability and blood-brain barrier (BBB) permeability. In order to find out the exposure of GSH-derived ingredients, including GSH, cysteine (CYS), glutamate (Glu), glycine (GLY), CYS-GLY and γ-Glutamylcysteine (γ-GC) were systematically studied both *in vitro* and *in vivo*. The outcomes demonstrated that oral GSH not only increases the GSH and CYS levels in rat striatum and cortex, but also can decrease the rise of intracerebral Glu concentration caused by ischemia/reperfusion (I/R) surgery. Then the influence of GSH on BBB was investigated via measuring IgG leakage, intracerebral endotoxin and tight junction proteins. All indicators showed that GSH-dosing can repair the destroyed BBB. This should be noted that oral GSH greatly enhances the exposure of GSH, CYS, CYS-GLY and γ-GC in rat duodenum, jejunum, ileum and colon. Accumulating evidence reveals a close linkage of brain injury and gastrointestinal dysfunction. Our findings further suggested that oral GSH significantly improves intestinal inflammatory damage and barrier disruptions. In conclusion, oral GSH can have a direct therapeutic role in brain injury by stabilizing intracerebral levels of GSH, CYS and Glu. It can also play an indirect therapeutic role by enhancing the intestinal exposure of GSH, CYS, CYS-GLY, γ-GC and improving intestinal barrier disruptions.

Keywords: Glutathione, Ischemia/reperfusion, Blood-brain barrier, GSH-derived ingredients, Intestinal barrier

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Significance statement

The authentic active ingredients and action sites of exogenous GSH in the treatment of ischemic brain injury are still unclear. We uncovered that oral exogenous GSH not only stabilizes intracerebral levels of GSH, CYS and Glu to act on brain injury directly, but also can exert an indirect therapeutic role by improving intestinal barrier disruptions. These findings have great significance for revealing the therapeutic effect of GSH on ischemic brain injury and promoting its further development and clinical application.

Introduction

Ischemic brain injury remains a leading cause of morbidity and mortality worldwide with a lack of therapeutic regime to prevent cell death and regenerate damaged cells (Kuroda et al., 1996; Siesjö et al., 1999; Kharrazian, 2015; Kahl et al., 2018). Oxidative stress and mitochondrial impairment are known to be early events in ischemic brain injury (Lin et al., 1993; Mustafa et al., 2013). BBB is also considered a major impediment to treatment of CNS diseases (Moskowitz et al., 2010; Birgit et al., 2013; Jamieson et al., 2017) . Recently, an increasing number of studies indicated a bidirectional route of communication between the gastrointestinal (GI) tract and the CNS, that is termed as the 'gut-brain axis' (Filpa et al., 2016). The degree of intestinal barrier disruption, such as increased intestinal permeability, proinflammatory factors and endotoxemia, positively correlates with the severity of cerebral ischemia/reperfusion (I/R) injury (Faries et al., 1998). Therefore, the causes and consequences of cerebral I/R injury injuries are complex and serious, thus an immense needs to develop effective therapeutic strategies for cerebral I/R injury via reducing oxidative damage and/or protecting the integrity of blood-brain and intestinal barriers.

GSH (L-c-glutamyl-L-cysteinylglycine), an intracellular thiol tripeptide present in all mammalian tissues, plays a crucial role in antioxidation and other some metabolic functions, but it is always depleted during the inflammatory responses (Yang et al.; Pallardó et al., 2009; Nosareva et al., 2017; Shi et al., 2017). Some clinical studies demonstrate that some of the neurological disorders, like Alzheimer's diseases (AD), Parkinson's diseases (PD), and strokes, are associated with decreased GSH levels (Merad-Boudia et al., 1998; Klaus et al., 2006; Ansari and Scheff, 2010; Cojocaru et al., 2013). Besides, the GSH's γ-peptide linkage can be hydrolyzed to CYS-GLY and Glu by the intestinal γ-glutamyltransferase, and the CYS-GLY can be further cleaved to generate CYS and GLY (Hanigan and Ricketts, 1993; Meister, 1994). Interestingly, most degradation products of GSH have been reported to be associated with oxidative stress and brain damage. For instance, depletion of CYS may cause oxidative stress, which can

be associated with neurodegenerative disorders such as Huntington's disease, amyotrophic lateral sclerosis, AD and PD (Vandiver et al., 2013; Paul et al., 2014; Scheltens et al., 2016; Paul et al., 2018). The agents which stimulate synthesis of GSH from CYS can inhibit apoptosis induced by oxidative stress (Ratan et al., 1994; Paul et al., 2018) . The amino acid GLY, another degradation product of GSH, is a major inhibitory neurotransmitter that bind to GLY receptors to inhibit postsynaptic neurons (Lynch, 2009). Recently, Liu *et al.* found that the injection of GLY could indirectly reduce ischemia-induced neuronal death, brain damage and functional recovery (Liu et al., 2019). As another degradation product of GSH, Glu is reported as a principal excitatory neurotransmitter in the nervous system. Excessive release of Glu into extracellular spaces may lead to excitotoxic neuronal damages, thus it is important that Glu concentrations should be strictly controlled in brain (Nishizawa, 2001; Ji et al., 2019). In 2015, Song *et al.* reported that exogenous intravenous injection of GSH attenuated cerebral infarct volume after ischemic stroke by promoting the PI3K/Akt pathway and inhibiting the translocation of FOXO3 into the nucleus (Song et al., 2015). However, GSH cannot pass the BBB easily. The poor ability to permeate BBB and unstable structural characteristics of GSH make its therapeutic effects in ischemic brain injuries disputed. Many doubts should be clarified as follows: (1) Does oral GSH also play therapeutic roles in brain injury? (2) What are GSH-related active ingredients? (3) Does GSH act directly on the brain or indirectly on the peripheral?

In the present study, the therapeutic effects of oral GSH on ischemic brain injuries were first investigated in I/R model rats (*in vivo*) and oxygen-glucose deprivation / reperfusion model cells (*in vitro*). The results suggested that oral GSH could improve the neurological deficit score, infarct size histological lesions, pro-inflammatory cytokines and BBB disruption caused by I/R surgeries. The intracerebral distribution of GSH-derived ingredients showed that I/R surgeries could lead to a decrease of GSH & CYS in striatum and cortex of injured cerebral hemisphere where oral GSH could significantly enhance the GSH and CYS levels. The increased intracerebral Glu caused by I/R surgeries could also be reduced by oral GSH. More importantly, oral GSH could significantly enhance the

intestinal exposure of CYS, CYS-GLY and γ-GC which can improve intestinal inflammatory damages and barrier disruptions by decreasing pro-inflammatory cytokines and up-regulating intestinal tight junction proteins.

Material and Methods

Materials

GSH (Lot No. B141015) was kindly supplied by Kunming Jida Pharmaceutical Co., LTD (Kunming, Yunnan, China). Cell counting kit 8 (CCK-8) reagents were purchased from Beyotime Institute of Biotechnology (Shanghai, China). [$^{3\text{-}13}$C]-L-GSH, CYS, CYS-GLY, γ-GC, Glu, GLY, captopril (CAP), N-ethylmaleimide (NEM) and 2'7 '-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Buthionine sulfoximine (BSO) were purchased from Aladdin (Shanghai, China).

Animals and Treatments

Animals Male healthy Sprague-Dawley (SD) rats (aged 8~9 weeks, weighing 220~250g) were purchased from the Sipper-BK laboratory animal CO., Ltd (Shanghai, China). The rats were housed under controlled condition (25℃, 55%-60% humidity and 12-hour light/dark cycle) with free access to laboratory food and water. All studies were strictly in compliance with animal care laws and guidelines, and approved by the China Pharmaceutical University Animal Care and Use Committee.

Preparation of I/R model rats The rats were anaesthetized by intraperitoneal injection of 10% chloral hydrate (0.3mL/100g). A silicone rubber coated filament was used to occlude the left middle cerebral artery (MCA) via the external carotid artery into the internal carotid artery and MCA. The correct position was confirmed by meeting a mild resistance, approximately 21 mm beyond the common carotid artery bifurcation. After middle cerebral arterial occlusion (MCAO) for 2h, the occluding filament was withdrawn to allow reperfusion. Then the rats were intragastrically administrated with GSH (250mg/kg) or

saline (vehicle).

Neurological assessments and measurement of infarct sizes Neurological function was performed by behavioral test according to previous reports (Longa et al., 1989). The modified scoring system is divided into five scales (0 - 4) with higher scores indicating severer neurological injury. Rats were sacrificed at 24 h after I/R surgeries. The collected brains were sectioned into consecutive 2 mm thick coronal slices with a cryomicrotome (Leica CM1950, Nussloch, GER), and then immersed in 1% TTC medium for 10 min at 37 ℃. Image-J 1.8.0 software was used to calculate infarct sizes.

Measurement of pro-inflammatory cytokines in rats brain and ileum To evaluate inflammatory injury caused by I/R surgeries and the protective functions of GSH, the levels of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) in rats brain and ileum were determined using the corresponding commercial ELISA kits (Excellbio Technology, Shanghai, China) following the manufacturer's instructions. The results were calibrated by protein concentration.

Measurement of tight junction proteins in rats brain and ileum To assess the expressions of tight junction proteins, rats brain and ileum were homogenized in ice-cold PBS buffer and then centrifuged at 12000×g for 10 min at 4 ℃. The protein expressions of ZO-1, Claudin-5 and Occludin in supernatants were measured by using commercial ELISA kits (Shanghai Enzyme-linked Biotechnology, Shanghai, China).

Histopathological analysis of rats brain and intestine Fresh brains and intestines of rats were fixed in 4% paraformaldehyde for 2 days. Then the fixed tissues were sectioned and stained using a picrosirius red solution for 1 h according to the protocols of histopathological technique. After washing 3 times by acidified water, tissue sections were counterstained with carazzi’s hematoxylin.

Measurement of IgG leakage in rat brain BBB disruption was examined by determining IgG leakage of in rats brains. In this process, brain tissues were cut into 50 μm thickness and then attached to glass slides following to incubated with 3% H_2O_2. Then the brain

sections were incubated with antibody against IgG (1:500, Acam) for 1 h at room temperature and then labeled by diaminobenzidine (DAB).

Measurement of endotoxin At 24h after I/R surgeries, venous blood was collected into heparinized pyrogen-free tubes. Brain tissues were homogenized in 10-folds volume of pyrogen free homogenization buffer. Samples were centrifuged at 12000×g for 5min and then frozen at -80℃. Endotoxin concentration was determined by commercial kits (Horseshoe Reagent Biotechnology, Xiamen, China).

Cell culture and Treatments

Cell culture The human cerebral microvascular endothelial cell (hCMEC/D3) lines were purchased from Biotechnology Co., Ltd. Shanghai enzyme research (Shanghai, China), and cultured in Dulbecco's Modified Eagle Media (Gibco) containing 1% penicillin / streptomycin, 2 mM of L-glutamine and 10 % fetal bovine serum, and maintained in a humidified incubator (95% air and 5% CO_2) at 37 ℃.

Oxygen-glucose deprivation/reperfusion (OGD/R) model Multiple steps were involved in establishing OGD/R model: (i) washed the hCMEC/D3 cells seeded in plates using fresh glucose-free medium for 3 times; (ii) replaced fresh glucose-free medium with glucose-free DMEM medium; (iii) put the cell plates into a hypoxic chamber which is continuously fed with 95% (volume fraction) N_2 , 5% CO_2 and less than 1% O_2, (iv) after 12 h later, all the cell plates were took out and were replaced with DMEM medium containing glucose with 95% air and 5% CO_2 for reoxygenation of cells for 1 h. In addition, cells in the control group were cultured in DMEM medium containing glucose and placed under normal culture conditions for the same periods of time.

Cell treatments For GSH-dosed group, 2 mM of GSH was previously dissolved in the culture medium at pH 6.8. The hCMEC/D3 cells were treated with 2mM GSH during OGD/R. For BSO-dosed group, 2 mM of BSO (the inhibitor of GSH synthetase) was previously dissolved in the culture medium with 0.02% DMSO. Then the cells were treated with 2mM BSO during OGD/R.

Cell viability and scratch assays Cell viability was measured by adding CCK-8 reagent according to manufacturer instructions strictly. The absorbance intensity was measured at 450nm using a microplate reader. The results were expressed by the fold change relative to control cell viability. For scratch assays, the hCMEC/D3 cells were seeded in 6-well plates in DMEM medium for 24 h. After 90% confluence, cells were scratched along the diameter of the well. Then the cells were washed twice with serum-free medium and cultured in normal or OGD/R condition. Images were acquired at 12 and 24 h using a microscope system.

Measurement of intracellular reactive oxygen species (ROS) Oxidative stress was characterized by intracellular ROS levels using DCFH-DA staining assay. Briefly, hCMEC/D3 cells were seeded in 96-well plates at a density of 1×10^5/ well. Cells were washed gently by pre-warmed sterilized PBS, and then incubated in serum-free DMEM containing 10 μM DCFH-DA for 1 h at 37℃. After washing the cells 3 times with PBS, images were acquired on laser scanning confocal microscopy. The fluorescence intensity of ROS was determined by a multifunctional fluorescence microplate reader which wavelengths were set at 488 and 525 nm for excitation and emission, respectively.

Quantitative analysis of GSH, CYS, CYS-GLY, γ-GC, [$^{3\text{-}13}$C]-L-GSH and [$^{3\text{-}13}$C]CYS-GLY in biological matrices using LC-MS/MS

Pretreatment of tissue specimens The fresh rat tissues were homogenized in 10-fold volume of homogenization buffer containing 6 mg/mL of Tris, 0.2 mg/mL of serine, 1.24 mg/mL of boric acid, 4μg/mL of acivicin and 7.76 mg/mL of NEM. After adding 10 μL of internal standard solution (5 μg/mL of CAP-NEM) into 50 μL of tissue homogenization, derivation was lasted for 1 h under the light proof environment. Then 200μL of methanol was added to precipitate the proteins. After vortex-mixing for 5 min and centrifuging at 30000×g for 10 min at 4℃, 5μL of supernatant was analyzed using LC-MS/MS 4000 system (SCIEX, MA, USA).

Pretreatment of cell specimens The cells were washed gently with serum-free medium

three times. After adding 100 μL of H_2O, cell suspension was collected after repeated freezing and thawing for 3 times. In 50 μL of cell suspension, 10 μL of derivatization internal standard (5 μg/mL of CAP-NEM) and 50 μL of derivative reagent were added, and derivation was lasted for 1 h under the light proof environment. Methanol (200μL) was added to remove the proteins, and 5μL of supernatant was analyzed using LC-MS/MS.

LC-MS/MS conditions Chromatographic separation was carried out on a Sepax Bio-ODS SP column (4.6*150mm, 5μm) under a gradient program. The mobile phase A: consists of H_2O containing 0.1% formic acid and 2 mM ammonium formate. Mobile phase B: methanol. The gradient program was as follows: 0 min, 10% B →0.2 min, 10% B → 0.4 min, 50% B → 4 min, 50% B → 4.5 min, 10% B → 6min, 10% B. The MS parameters were optimized as follows: ion spray voltage, 4500V; ion source gas 1, 60 psi; curtain gas, 10 psi; ion source gas, 50 psi; collision gas, 6 psi; source temperature, 550 °C. The optimized multiple reaction monitoring (MRM) parameters are listed in **Table S1**.

Quantitative analysis of GLY and Glu in biological matrices using LC-Q-TOF MS

Pretreatment of tissue specimens The fresh rat tissues (50 mg) were homogenized in 500 μL of H_2O. After centrifuging at 30000 ×g for 5 min, 400 μL of methanol containing ^{13}C-glutamine (15 μg/mL) was added to 100 μL of the supernatant, followed by vortex-mixing for 5 min and centrifuging at 30000×g for 10 min at 4℃. The supernatant (300 μL) was evaporated to dryness in a rotary evaporator, and the residue was reconstituted in 150 μL of H_2O.

Pretreatment of cell specimens A total of 800 μL of methanol containing 1.5 μg/mL of ^{13}C-glutamine was added to 200 μL of cell suspension. After vortex-mixing for 5 min and centrifuging at 30000×g for 10 min, 300 μL of supernatant was evaporated to dryness in a rotary evaporator and the residue was reconstituted in 100 μL of H_2O.

LC-Q-TOF MS conditions Chromatographic separation was carried out on a Waters Bridge Amide (3.5μm, 4.6×100mm) column under a gradient program. The flow rate was

0.4mL/min. The mobile phase A consists of H_2O containing 5 mM ammonium acetate and 5 % acetonitrile, and pH was adjusted to 9.0 with aqueous ammonia, while mobile phase B is acetonitrile. The gradient program was set as follows: 0 min, 15% B → 3 min, 15% B → 6 min, 70% B → 15 min, 98 % B → 18 min, 98% B → 19 min, 15% B → 26 min, 15% B. MS analysis was performed using AB Sciex 5600 Triple TOF MS which operated in negative ionization mode with a DuoSpray ion source (Concord, Ontario, Canada). The source conditions were set as follows: ion-spray voltage floating -4.5 kV, declustering potential 70 V, turbo spray temperature 500 ◦C, nebulizer gas (Gas 1) 50 psi, heater gas (Gas 2) 60 psi, curtain gas 30 psi. The accumulation time for MS^1 full scan was 100 ms for scanning a mass range from 100 to 1000 Da. The accumulation time for each IDA experiment was 50 ms, and the CE was set to 35 V with a CE spread of 15 V in high sensitivity mode. All the parameters were controlled were run by Analyst TF 1.7 software, and data were processed by MultiQuant 2.0 Software (Sciex, Concord, Ontario, Canada).

Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA) using Graph Pad Prism (version 6.0,San Diego, CA, USA). Differences among means were analyzed by Post hoc analysis or Dunnett's multiple comparisons test. Statistically significant difference was considered for $P< 0.05$.

Results

Investigation of the therapeutic effect of oral GSH on I/R in rats

The rats were intragastrically administrated with GSH (250 mg/kg) or saline (vehicle) after I/R surgeries, and brain infarct volume was estimated using TTC staining assay. As shown in **Figure 1A**, cerebral infarcts were pronounced after I/R surgeries, and the ischemia degree of GSH-dosed rats was significantly lower than that of I/R model rats. Infarct size could be decreased from 31.21 % to 9.62 % by oral GSH (**Figure 1B**). Meanwhile,

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intragastric administration of GSH greatly decreased the neurological deficit score of I/R model rats (**Figure 1C**). The results of hematoxylin-eosin (H&E) staining of coronal section showed that I/R model rats brain, mainly including striatal and cortical regions, had serious histological lesions, which were characterized by incomplete cortical structure, necrotic neurons and broken cerebrovascular tissues. The histological lesions of I/R model rats were effectively alleviated by intragastric administration of GSH. (**Figure 1D**). Pro-inflammatory cytokine (TNF-α, IL-1β and IL-6) levels in rat brain were determined to further investigate the therapeutic effect of oral GSH on ischemic brain injury. As shown in **Figure 1E** to **1G**, levels of TNF-α, IL-1β and IL-6 in the I/R rat brain were significantly higher than those of sham-operated group (one-way ANOVA, $P < 0.01$). GSH-dosing could significantly reverse the up-regulation of pro-inflammatory cytokine induced by I/R surgeries.

Investigation of the therapeutic effect of exogenous GSH in hCMEC/D3 cells

The OGD/R hCMEC/D3 cell model was built to further confirm the therapeutic effects of exogenous GSH on ischemic brain injury. As shown in **Figure 2A**, viability of hCMEC/D3 cells could be significantly enhanced by exogenous GSH (*P<0.01*), while BSO reduced the cellular viability significantly (*P<0.05*). The following experiment was to investigate the effect of GSH on cell migration using scratch assay. As shown in **Figure 2B** and **Figure S1**, exogenous GSH could significantly increase the migration rate of hCMEC/D3 cells cultured in normal conditions, while BSO-dosing reduced the migration of cells to some extent. In addition, exogenous GSH could also significantly increase the migration rate of the OGD/R-model hCMEC/D3 cells at 12 h (*P<0.01*), while BSO-dosed further inhibited cell migration. Furthermore, the influence of GSH on the oxidative stress levels was assessed, and the intracellular ROS was labeled with DCFH-DA probe. Clearly, the fluorescence intensity of OGD/R-model cells was much higher than that of the conventional cells, and BSO-treated cells had higher fluorescence intensity than that of model group cells. GSH could dose-dependently reduce the fluorescence intensity of OGD/R-model cells within the dose range from 0.5 to 5 mM (**Figure 2C** and **2D**). Thus,

exogenous GSH could effectively repair hypoxia-induced cell damage.

Distribution of GSH-derived ingredients in hCMEC/D3 cells

OGD/R model hCMEC/D3 cells were treated with 2 mM of GSH or BSO for 1, 6 and 12 h, respectively. Then the intracellular concentrations of GSH-derived ingredients, including GSH, CYS, Glu, GLY, CYS-GLY and γ-GC, were measured using LC-MS/MS and LC-Q-TOF MS. As shown in **Figure 3A**, GSH levels in the cells modeled by OGD/R for 1 h were significantly higher than those in conventional cells. No significant change was found in GSH concentration after treated with 2 mM GSH for 1 h, while BSO treatment reduced intracellular GSH concentration significantly (*P<0.05*). By contrast, GSH levels in the cells modeled by OGD/R for 6 h and 12 h were significantly lower than those in conventional cells. After treated with 2 mM of GSH for 6 h or 12 h, the intracellular GSH concentrations were much higher than those in OGD/R model cells. Exposure to BSO for 6 h or 12 h significantly reduced the intracellular GSH concentrations (*P<0.01*). Then the intracellular concentrations of the degradation products of GSH were measured. As shown in **Figure 3B**, the concentrations of CYS in control, OGD/R-model, OGD/R-model+GSH and OGD/R-model+BSO groups had no significant difference at 1 h and 6 h. However, CYS levels in the OGD/R-model cells collected at 12 h were significantly higher than those in the control group, which indicating the reduced ability of CYS to synthesize GSH in OGD/R-model cells. In contrast, the decreased CYS levels in GSH-treated cells demonstrated that GSH could restore the synthesis of CYS by alleviating hypoxia-induced cell damage. The exposure of Glu was shown in **Figure 3C**. The intracellular Glu concentrations in hCMEC/D3 cells modeled by OGD/R for 1, 6 and 12 h were all significantly lower than those in control group cells. GSH treatment could greatly increase the Glu levels, while BSO-dosing reduced the intracellular Glu exposure at 12 h. The other degradation products, including GLY, CYS-GLY and γ-GC, were also quantitatively analyzed, and the results elucidated that GSH treatment had no significant effect on intracellular concentrations of GLY and CYS-GLY (**Figure 3D and 3E**). The concentration of γ-GC in hCMEC/D3 cells was lower than the minimum quantitative limit. In conclusion,

exogenous GSH mainly affected the intracellular concentrations of GSH, CYS and Glu.

In order to further investigate the influence of exogenous GSH on intracellular GSH levels, OGD/R model hCMEC/D3 cells were incubated with 200 μM of [$^{3\text{-}13}$C]-L-GSH, and the intracellular and [$^{3\text{-}13}$C]-L-GSH and GSH concentrations were both measured using LC-MS/MS. As shown in **Figure 3F** and **3G**, the intracellular concentration of [$^{3\text{-}13}$C]-L-GSH was about 1/50 of that of GSH, and was much lower than 200 μM. Thus, GSH had low uptake capability in hCMEC/D3 cells, and endogenous GSH level in GSH-dosed hCMEC/D3 was much higher than that of exogenous GSH.

Distribution of GSH-derived ingredients in I/R model rat brains

The intracerebral distribution of GSH-derived ingredients in sham-operated, I/R-model and I/R+GSH rats was measured to search the active ingredients and action sites of oral GSH. Clearly, the intracerebral GSH levels in I/R-model rats were significantly lower than those of sham-operated group, and GSH-dosing could significantly reverse the reduction of GSH concentration caused by I/R surgeries (**Figure 4A**). The changing pattern of CYS was almost as same as that of GSH, and GSH-dosing could enhance the intracerebral CYS levels in I/R-model rats (**Figure 4B**). However, the changing pattern of Glu was contrary to that of GSH. I/R surgeries significantly increased the intracerebral Glu exposure, and GSH-dosing could decrease the Glu concentrations in I/R model rat brain (**Figure 4C**). The intracerebral concentrations of CYS-GLY and GLY among sham-operated, I/R-model and I/R+GSH groups had no significant difference (**Figure 4D** and **4E**). Besides, the concentration of γ-GC was too low to be detected.

The concentrations of GSH and CYS in different brain regions, including striatum, cortex, hippocampus and hypothalamus, were further quantitatively analyzed to seek the specific action sites of GSH. As shown in **Figure 4F** and **4G**, the GSH concentrations in striatum and cortex of left injured (ipsilateral) hemisphere were significantly lower than those of sham-operated group, and intragastric administration of GSH could greatly increase the GSH concentrations in ipsilateral striatum and cortex. By contrast, the GSH concentrations in ipsilateral hippocampus were significantly higher than those of

sham-operated group, and intragastric administration of GSH could greatly reverse the rise of GSH concentration caused by I/R surgeries (**Figure 4H**). In addition, exogenous GSH could significantly enhance the GSH concentration in hippocampus of I/R rats (**Figure 4I**). In right non-injured (contralateral) hemisphere, the GSH concentrations had no significant difference among the sham-operated, I/R-model and I/R+GSH groups. Similar to GSH, the CYS concentrations in ipsilateral striatum and cortex of I/R rats were significantly lower than those of sham-operated group rats, and intragastric administration of GSH greatly increased the GSH concentrations in these two regions. In contralateral cerebral hemisphere, GSH increased striatal CYS level, but had no effect on cortical CYS concentrations (**Figure 4J** and **4K**). In addition, the CYS concentrations in ipsilateral hippocampus were decreased by I/R surgeries, and GSH-dosing could increase the CYS to some extent although the difference between I/R and I/R+GSH groups was not significant. Curiously, GSH-dosing decreased the CYS levels in the contralateral hippocampus (**Figure 4L**). Besides, the CYS levels in hypothalamus of I/R group were much higher than those of sham-operated rats, and GSH-dosing could further increase the CYS concentration in hypothalamus. In conclusion, the therapeutic effect of oral GSH on ischemic brain injury might rely primarily on the concentration increase of GSH and CYS in ipsilateral striatum and cortex, and reduction of intracerebral Glu exposure might also be one of the mechanisms of GSH to treat brain injury. Thus, oral GSH could play a direct role in the treatment of brain injury by stabilizing intracerebral levels of GSH, CYS and Glu.

The influence of exogenous GSH on the distribution of GSH in rat tissues

The intra-tissue concentrations of GSH-derived ingredients in rat plasma, heart, liver, stomach, duodenum, jejunum, ileum, colon and kidney were measured to investigate the influence of exogenous GSH on the distribution of GSH-derived ingredients. As shown in **Figure 5A**, intragastric administration of GSH could significantly increase the concentrations of GSH in I/R model rat plasma collected at 7 and 24h. Both I/R surgeries and oral exogenous GSH had no obvious effect on GSH level in rat heart, liver, kidney and

stomach (**Figure 5B**~**5E**). However, I/R surgeries and exogenous GSH had great effects on intestinal GSH level (**Figure 5F – 5I**). For instance, intragastric administration of GSH increased the duodenal GSH level of sham-operated rats by 10 times. The GSH levels in duodenum of I/R+GSH group rats collected at 7 and 24h were about 5 and 18 times higher than those in the I/R model group, respectively. Besides, intragastric administration of GSH could greatly enhance the GSH levels in rat jejunum, ileum and colon of both sham-operated and I/R model groups.

The intra-tissue concentrations of CYS, CYS-GLY, γ-GC, Glu and GLY were also measured, and the results were shown in **Figure S2** to **Figure S6**. Intragastric administration of GSH could significantly increase the concentrations of CYS in I/R model rat plasma and duodenum. GSH-dosing could also greatly enhance the CYS levels in I/R model rat jejunum and ileum collected at 7 h, while the effect of exogenous GSH on CYS level in other tissues was not significant (**Figure S2**). The analysis results of CYS-GLY showed that the CYS-GLY concentrations in rat plasma, liver, kidney and heart almost could not be altered by GSH-dosing. Nevertheless, intragastric administration of GSH significantly increased the concentrations of CYS-GLY in duodenum, jejunum, ileum and colon of sham-operated and I/R model rats (**Figure S3**). The exposure of γ-GC was also quantitatively analyzed although its concentrations were extremely low, and the γ-GC levels in the plasma and intestine could also be greatly enhanced by oral exogenous GSH (**Figure S4**). The Glu and GLY concentrations in rat tissues were greatly higher than those of CYS-GLY, γ-GC and CYS, and the effect of oral exogenous GSH on the exposure of Glu and GLY was significantly lower than that of other ingredients (**Figure S5** and **S6**). In conclusion, intragastric administration of GSH could greatly enhance the intestinal CYS, CYS-GLY and γ-GC levels in both sham-operated and I/R model rats.

The influence of exogenous GSH on intestinal injury caused by I/R surgeries

Accumulating evidence reveals a close linkage of brain injury and gastrointestinal dysfunction. Our findings suggested that oral exogenous GSH greatly enhanced the concentrations of GSH, CYS, CYS-GLY and γ-GC in rat intestines. Herein, the effects of

I/R surgeries and GSH-dosing on the gastrointestinal dysfunction were investigated by observing pathological sections and measuring the levels of inflammatory factors and tight junction proteins. More precisely, the intestines of I/R model rats were characterized by severe accumulation of inflammatory cells, incomplete structure or even shedding partial villus, gland degeneration and crypt structure change (**Figure 6A**). Intragastric administration of GSH greatly improved the intestinal pathological features. Besides, the levels of IL-6, TNF-α and IL-1β in the I/R rat intestine were significantly higher than those of sham-operated group (one-way ANOVA, P <0.01), and GSH-dosing could significantly reverse the up-regulation of pro-inflammatory cytokines induced by I/R surgeries (**Figure 6B** to **6D**). In addition, the tight junction proteins in rat intestine were determined based on ELISA assay. As shown in **Figure 6E** to **6G**, the intestinal levels of ZO-1, claudin-5 and occluding proteins in I/R group were significantly lower than those of sham-operated group. I/R surgeries might lead to the destruction of intestinal barrier, and GSH-dosing could significantly reverse the down-regulation of intestinal ZO-1 and claudin-5 proteins. Thus, oral exogenous GSH could alleviate intestinal damage caused by brain injury by reducing intestinal inflammation and intestinal barrier.

The influence of oral exogenous GSH on BBB destruction after I/R surgeries

The integrity of BBB was investigated by determining cerebral IgG leakage, tight junction proteins and the levels of endotoxin. The results illustrated that there was no IgG leakage in the sham-operated rat brain. After 24 h of I/R surgeries, severe IgG leakage occurred on the left injured hemisphere, and GSH-dosing significantly ameliorated IgG leakage (**Figure 7A**). Besides, GSH-dosing dramatically reversed the down-regulation of intracerebral ZO-1 and claudin-5 proteins induced by I/R surgeries (**Figure 7B** to **7D**). The improved intestinal and blood-brain barrier disruption might alter the levels of endotoxin, which positively correlate with severity of brain injuries (Faries et al., 1998). Herein, the intracerebral endotoxin levels were determined to further elucidate the protective effect of GSH on intestinal and blood-brain barrier. As shown in **Figure 7E**, the intracerebral endotoxin level in I/R model rats was significantly higher than that in sham-operated group

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(*P<0.01*). Intragastric administration of GSH could greatly decrease the intracerebral endotoxin level of the I/R rats (*P<0.05)*. Similarly, I/R surgeries could lead to elevated plasma endomycin level while GSH-dosing dramatically reduced it in I/R model rat plasma **(Figure 7F**). Thus, oral exogenous GSH could significantly repair the damage of BBB caused by brain injury.

Discussion

Oxidative stress is associated with various CNS diseases such as stroke, AD, PD, *etc* (Merad-Boudia et al., 1998; Klaus et al., 2006; Ansari and Scheff, 2010; Cojocaru et al., 2013). As an intracellular thiol tripeptide present in all mammalian tissues, GSH plays a crucial role in cellular protection against oxidant damage. Reduction of GSH level *in vivo* may lead to the degeneration of dopaminergic neurons (Bilgin et al.; Li et al., 1997; Cadenas, 2004). In 2015, Song *et al.* reported that intravenous injection of exogenous GSH attenuated cerebral infarct volume after ischemic stroke by promoting the PI3K/Akt pathway (Song et al., 2015). The therapeutic effects of oral GSH were investigated in I/R model rats and OGD/R-model hCMEC/D3 cells at present study. The results demonstrated that intragastric administration of GSH could not only greatly improve the neurological deficit score, infarct size and histological lesions of I/R model rat, but also significantly reverse the up-regulation of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) induced by I/R surgeries. *In vitro* PD studies further suggested that exogenous GSH could dose-dependently increase the survival of hCMEC/D3 during OGD/R by increasing the migration rate of the OGD/R-model cells and suppressing the generation of ROS. BSO is an inhibitor of the rate-limiting enzyme, γ-glutamylcysteine synthetase, in GSH biosynthesis (Li et al., 1997). In addition to increasing intracellular ROS, BSO-treatment could lead to a decrease in the survival and migration rate of OGD/R model hCMEC/D3 cells. Thus, exogenous GSH could be used to treat ischemic brain injury. However, the effectiveness of oral GSH as a therapeutic agent for brain injuries may be limited because of its low bioavailability and poor ability to permeate BBB (Takujiro and Junichi, 2015). According to previous reports, the intestinal γ-glutamyltransferase could hydrolyze

GSH's γ-peptide linkage to produce CYS-GLY and Glu, and the CYS-GLY could be further cleaved to generate CYS and GLY (Hanigan and Ricketts, 1993; Meister, 1994). Interestingly, most degradation products of GSH, including CYS, GLY and Glu, have been reported to be associated with oxidative stress and brain damage. For instance, CYS participates in a wide variety of redox reactions due to its sulfur atom (Vandiver et al., 2013; Paul et al., 2014; Scheltens et al., 2016; Paul et al., 2018). Another degradation product, GLY, is a major inhibitory neurotransmitter that binds to glycine receptors to inhibit postsynaptic neurons (Lynch, 2009; Liu et al., 2019). Besides, excessive release of Glu into extracellular spaces is proved to cause excitotoxic neuronal damages, and the concentration of Glu in the brain should be strictly controlled (Nishizawa, 2001; Ji et al., 2019).

In order to elucidate the paradox between PK and PD of GSH, first we carried out studies on the distribution and uptake of GSH-derived ingredients, including CYS, GLY, Glu, CYS-GLY and γ-GC, in *in vitro* and *in vivo* models. *In vitro* studies suggested that exogenous GSH mainly enhanced the intracellular concentrations of GSH and Glu, yet almost had no significant effect on exposure of GLY, CYS-GLY and γ-GC in hCMEC/D3 cells. Intracellular CYS level increased significantly after OGD/R injury, GSH treatment could decrease the CYS levels by alleviating OGD/R -induced cell damage and restoring the synthesizing ability of CYS. To further investigate the uptake capacity of GSH, OGD/R model hCMEC/D3 cells were incubated with 200 μM of [$^{3\text{-}13}$C]-L-GSH. The results proved that the concentration of endogenous GSH was significantly higher than that of exogenous GSH. GSH had low uptake capability, which was consistent with its low bioavailability. The cerebral distribution of GSH-derived ingredients (GSH, CYS, GLY, GLY, CYS-GLY and γ-GC) showed that I/R surgeries could lead to decreased levels of GSH and CYS in rat striatum and cortex of injured cerebral hemisphere, and intragastric administration of GSH could significantly reverse this decline of GSH and CYS levels caused by I/R surgeries. We also found that I/R surgeries resulted in an increase in intracerebral Glu concentration. The main reason might lie in the increased permeability

of BBB, which always produce severe clinical consequences, such as vasogenic brain edema, hemorrhagic transformation and poor neurological outcomes (Xu et al., 2005; Moskowitz et al., 2010; Khatri et al., 2012). Our results also suggested that the increase of Glu exposure in I/R rat brain could be significantly reversed by oral GSH, which implied that exogenous GSH may have the function of repairing BBB. In fact, the leakage of serum proteins into brain parenchyma could be used to examine the integrity of BBB (Mann et al., 2016). Then we investigate the influence of GSH on BBB via determining the IgG leakage, intracerebral tight junction proteins, and the levels of endotoxin. All indicators showed that GSH-dosing had the function of repairing the destroyed BBB caused by I/R operation. Thus, oral exogenous GSH could play a direct role in the treatment of brain injury by stabilizing intracerebral GSH, CYS and Glu, repairing destroyed BBB, and down-regulating pro-inflammatory cytokines.

Although oral exogenous GSH could play a direct therapeutic role in brain injury, we cannot help wondering if GSH can also play an indirect therapeutic role due to its low bioavailability and poor ability to permeate BBB. The concentrations of GSH-derived ingredients in rat plasma, heart, liver, kidney, stomach, duodenum, jejunum, ileum and colon were measured to identify other possible action sites of oral GSH. Curiously, intragastric administration of GSH could significantly enhance the intestinal exposure of GSH-derived ingredients, including GSH, CYS, CYS-GLY and γ-GC. According to previous reports, GSH was formed from CYS by the enzymatic action of glutamate–cysteine ligase (GCL) which comprised of the catalytic subunit (GCLc) and the modulating subunit (GCLm) (Meister and Anderson, 1983; Lu, 2013; Takujiro and Junichi, 2015). The GCLc expression in rat liver, the main organ for GSH synthesis, was determined to elucidate the effect of exogenous GSH on GSH synthesis *in vivo.* As shown in **Figure S7**, the expression of GCLc in the rat liver was significantly decreased by I/R surgeries (*P<0.001*), and oral administration of GSH had no obvious effect on GCLc expression. Thus, the increase of GSH concentrations in rat brains and intestines was not achieved by increasing GSH synthesis. Accumulating evidence reveals a close linkage of brain injury and gastrointestinal dysfunction. The influence of the GI tract on the brain of

human has been noted since the nineteenth century, and the neuroinflammation hypothesis has been advocated since 2000s (A et al., 2019). The structure and function of the brain can be modulated by the gut, and conversely, the brain regulates the gut microenvironment and microbiota composition (R and TW, 2016; Zhao et al., 2018). Changes of the intestinal flora easily caused small intestinal immune dysfunction, which might suppress the transporting of IL-17-positive γδT cells and/or Th17 cells from the small intestine to the peripheral blood, and then reduce systemic inflammation after brain injury(C et al., 2016; Honda and Littman, 2016). Gastrointestinal dysfunction, including mucosal injury, barrier disruption, dysmotility and inflammation, caused by brain injury might be one of the causes of morbidity and mortality (Min et al., 2011; Olsen et al., 2013). Recently, resveratrol was reported to improve cerebral ischemia through decreasing the ischemia-induced transfer of cytokines (IL-17A, IL-23, IL-10, IFN-γ, and IL-4) from the small intestine to the blood via attenuating the small intestinal epithelial permeability by Dou *et al*(Dou et al., 2019). In this study, the effects of I/R surgeries and GSH-dosing on the gastrointestinal dysfunction were investigated by observing pathological sections and measuring the levels of inflammatory factors and tight junction proteins. Our findings suggested that intragastric administration of GSH could significantly reduce intestinal inflammatory damage and improve intestinal barrier disruption by decreasing pro-inflammatory cytokines and up-regulating intestinal tight junction proteins ZO-1 and claudin-5.

In summary, oral exogenous GSH can not only play a direct therapeutic role in brain injury by stabilizing intracerebral levels of GSH, CYS and Glu, but also have an indirect therapeutic role by enhancing the intestinal exposure of GSH, CYS, CYS-GLY, γ-GC and improving intestinal barrier disruptions.

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Authorship Contributions

Conceived the study: Yan Liang, Guangji Wang

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Generated reagents: Yangfan Xu, Huimin Guo, Lin Xie

Wrote or contributed to the writing of the manuscript: Yan Liang, Guangji Wang, Chong Chen, Qingqing Ding, Kangrui Hu.

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Footnotes

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Figure captions

Figure 1 Therapeutic effect of oral GSH on ischemic brain injury in rats. (A) Coronal sections of TTC-stained brains, (B) Cerebral infarct volume, (C) Neurology deficit score, (D) H&E staining of coronal section, (E) Levels of TNF-α, (F) Levels of IL-1β, (G) Levels of IL-6.

Figure 2 Therapeutic effect of oral GSH on ischemic brain injury in hCMEC/D3 cells. (A) The viability (%) of control, OGD/R-model, GSH-dosed and BSO-dosed cells. (B) The hCMEC/D3 cell migration, (C) Fluorescence image of intracellular ROS, (D) Fluorescence intensity of intracellular ROS.

Figure 3 Distribution of GSH-derived ingredients in hCMEC/D3 cells after being treated with 2mM GSH or 2mM BSO for 1, 6 and 12 h. (A) GSH, (B) CYS, (C) Glu, (D) GLY, (E) CYS-GLY, (F) The concentrations of [$^{3\text{-}13}$C]-L-GSH in hCMEC/D3 cells, (G) The concentrations of GSH in hCMEC/D3 cells.

Figure 4 The intracerebral distribution of GSH-derived ingredients in sham-operated, I/R-model and I/R+GSH rats. (A) GSH, (B) CYS, (C) Glu, (D) GLY, (E) CYS-GLY, (F) The concentrations of GSH in striatum, (G) The concentrations of GSH in cortex, (H) The concentrations of GSH in hippocampus, (I) The concentrations of GSH in hypothalamus, (J) The concentrations of CYS in striatum, (K) The concentrations of CYS in cortex, (L) The concentrations of CYS in hippocampus, (M) The concentrations of CYS in hypothalamus.

Figure 5 The distribution of GSH in rat tissues. (A) Plasma (B) Heart, (C) Liver, (D) Kidney, (E) Stomach, (F) Duodenum, (G) Jejunum, (H) Ileum, (I) Colon.

Figure 6 The influence of exogenous GSH on intestinal injury caused by I/R surgeries. (A) Pathological sections, (B) Levels of IL-6, (C) Levels of TNF-α, (D) Levels of IL-1β, (E) Expression of ZO-1, (F) Expression of claudin-5 proteins, (H) Expression of occludin proteins.

Figure 7 The influence of exogenous GSH on destruction of BBB integrity caused by I/R surgeries. (A) IgG expression in rat brain, (B) Expression of ZO-1 proteins, (C) Expression of claudin-5 proteins, (D) Expression of occludin proteins, (E) Levels of endotoxin in brain, (F) Levels of endotoxin in plasma.

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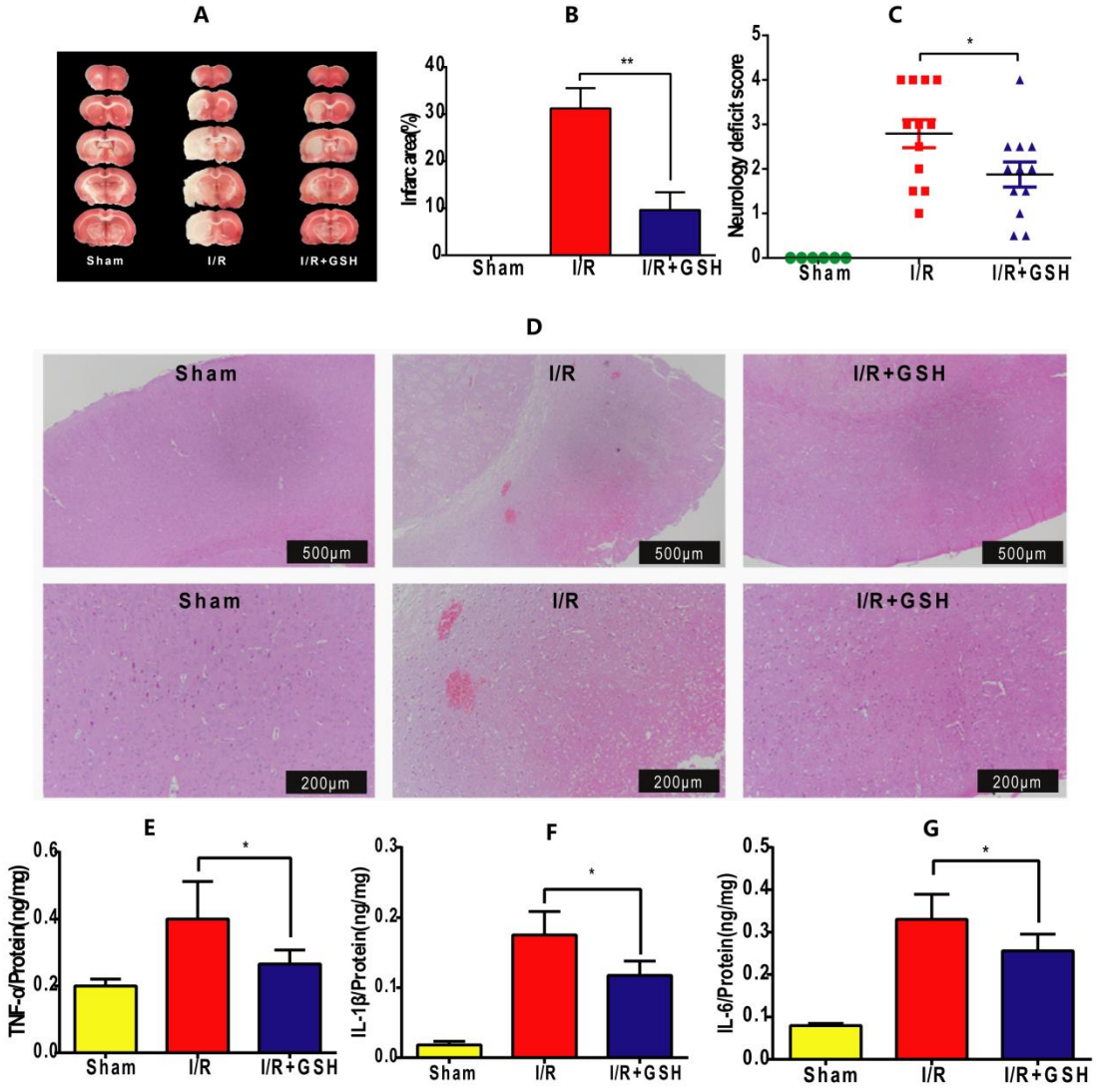

Figure 1

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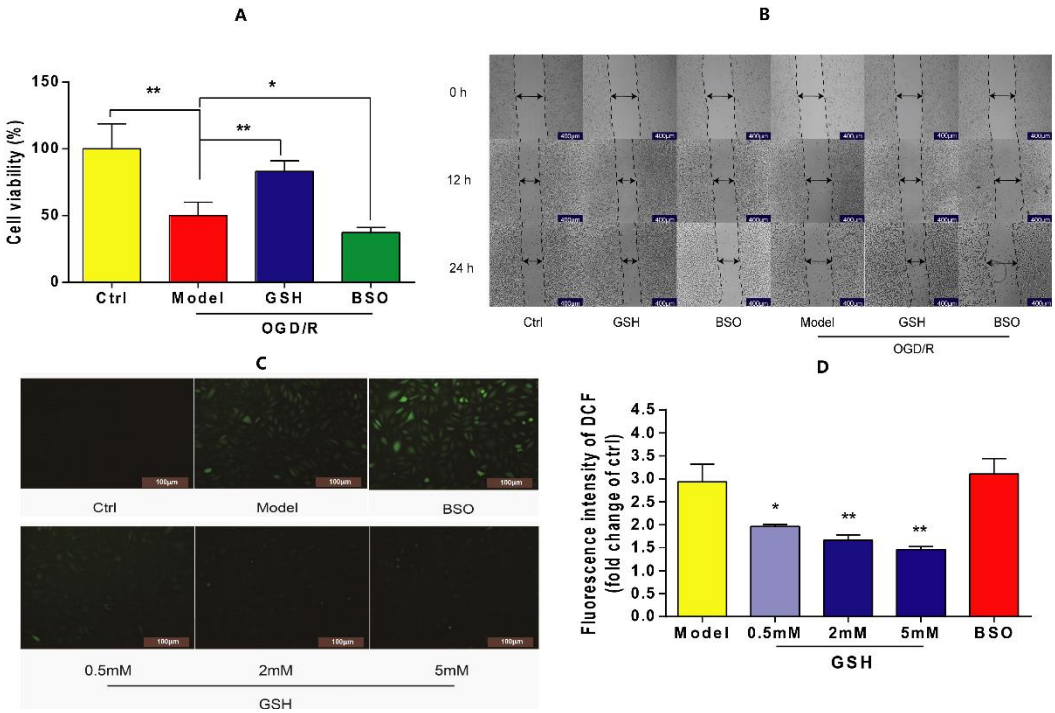

Figure 2

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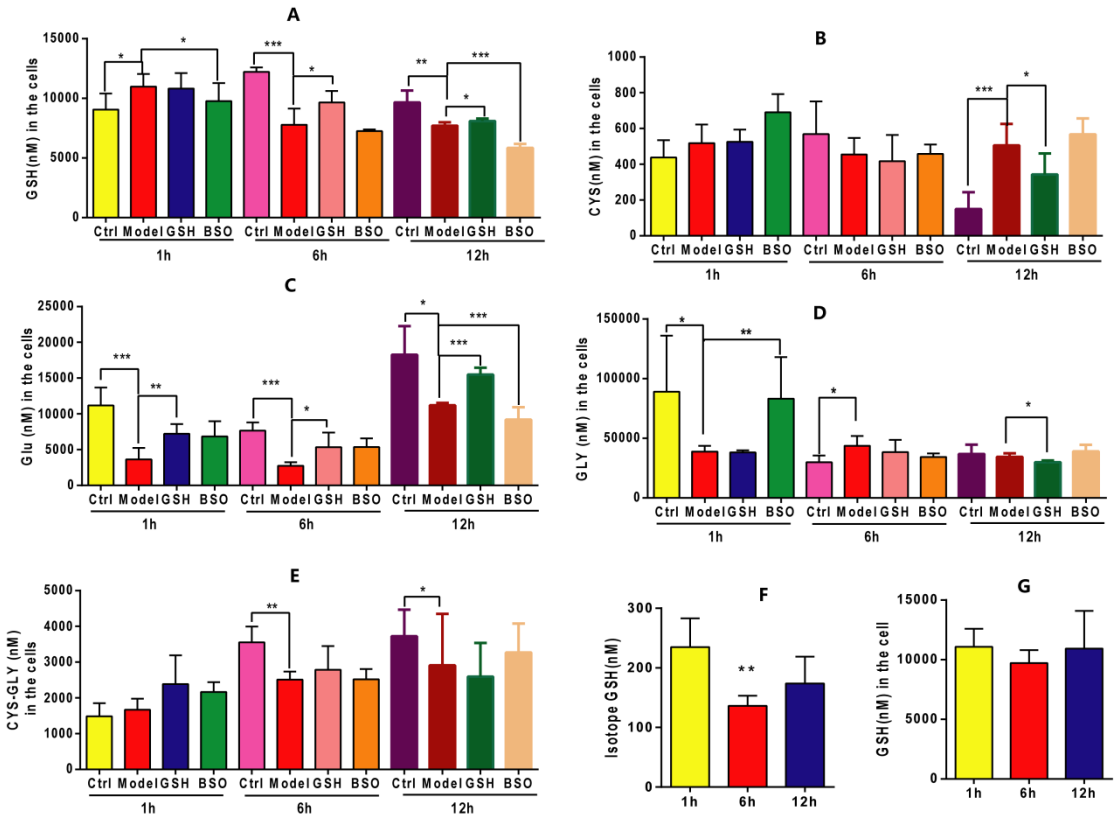

Figure 3

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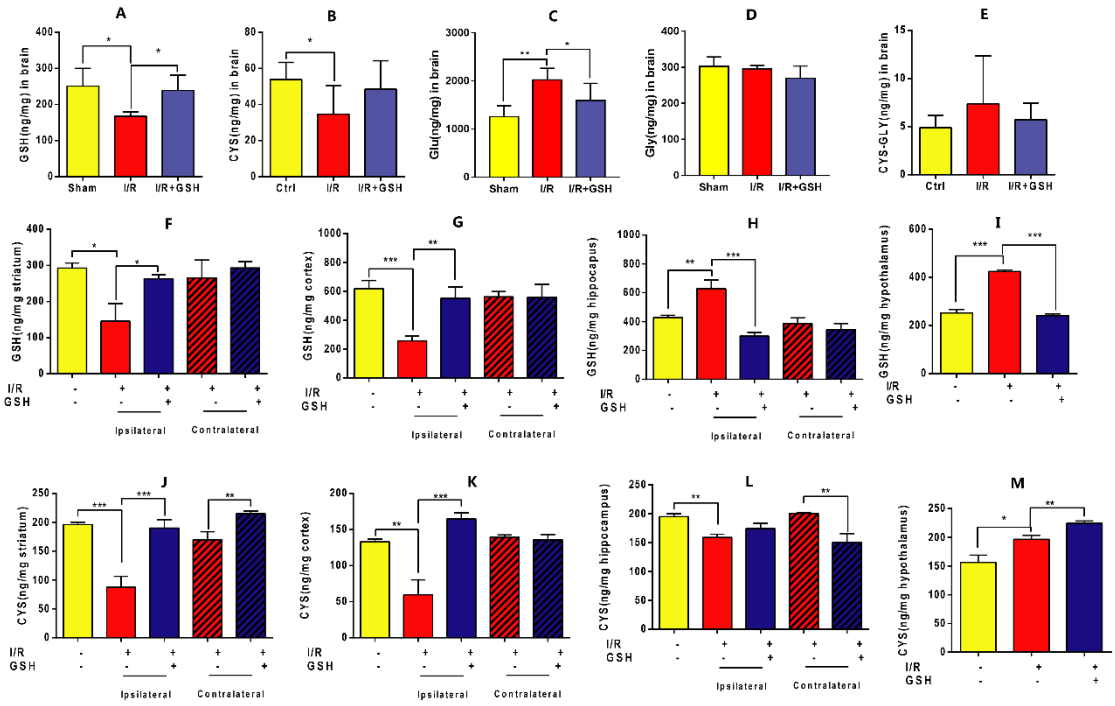

Figure 4

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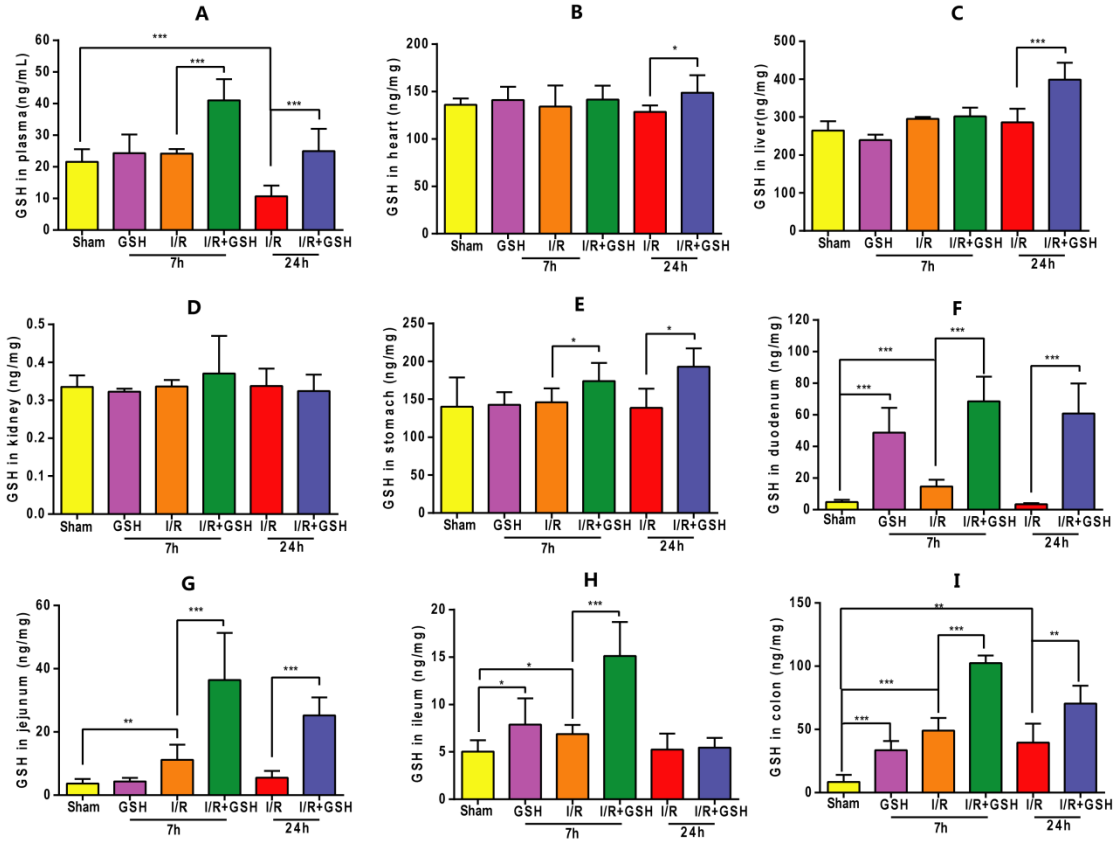

Figure 5

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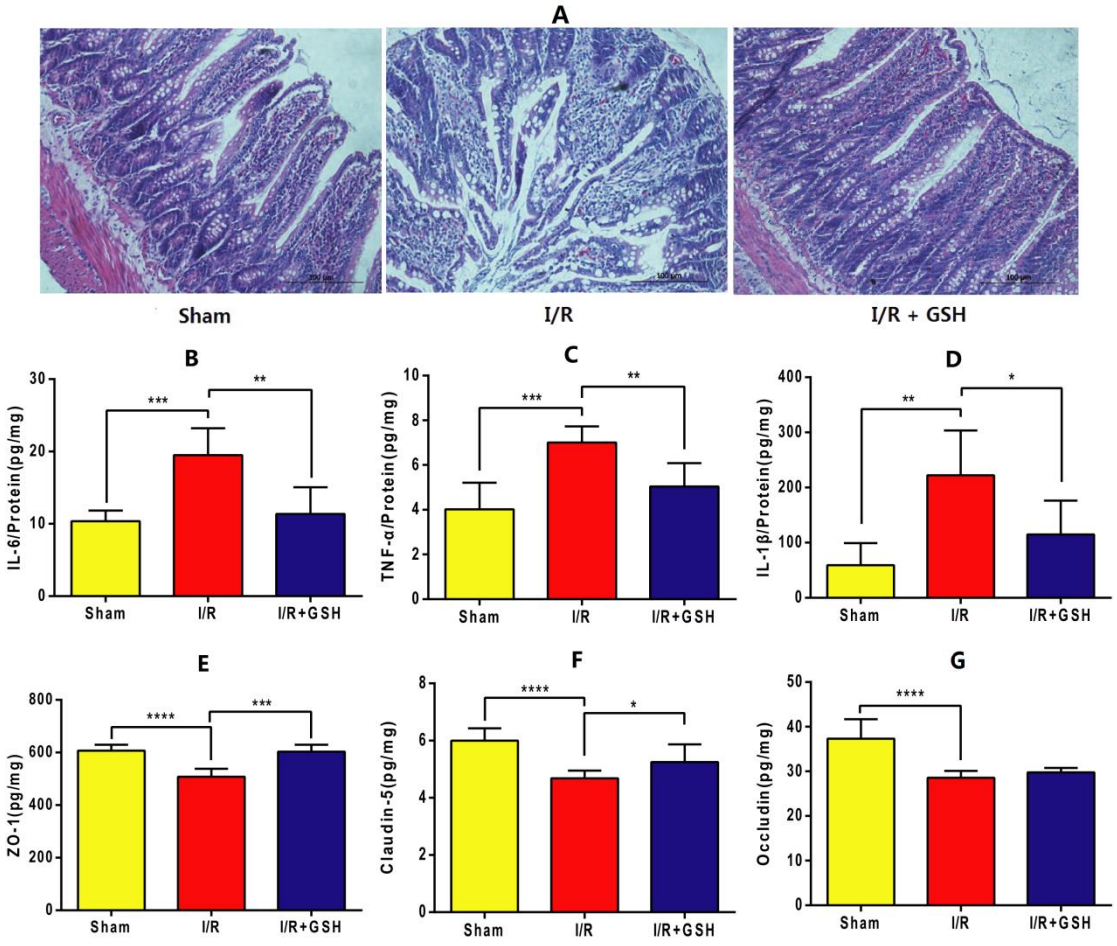

Figure 6

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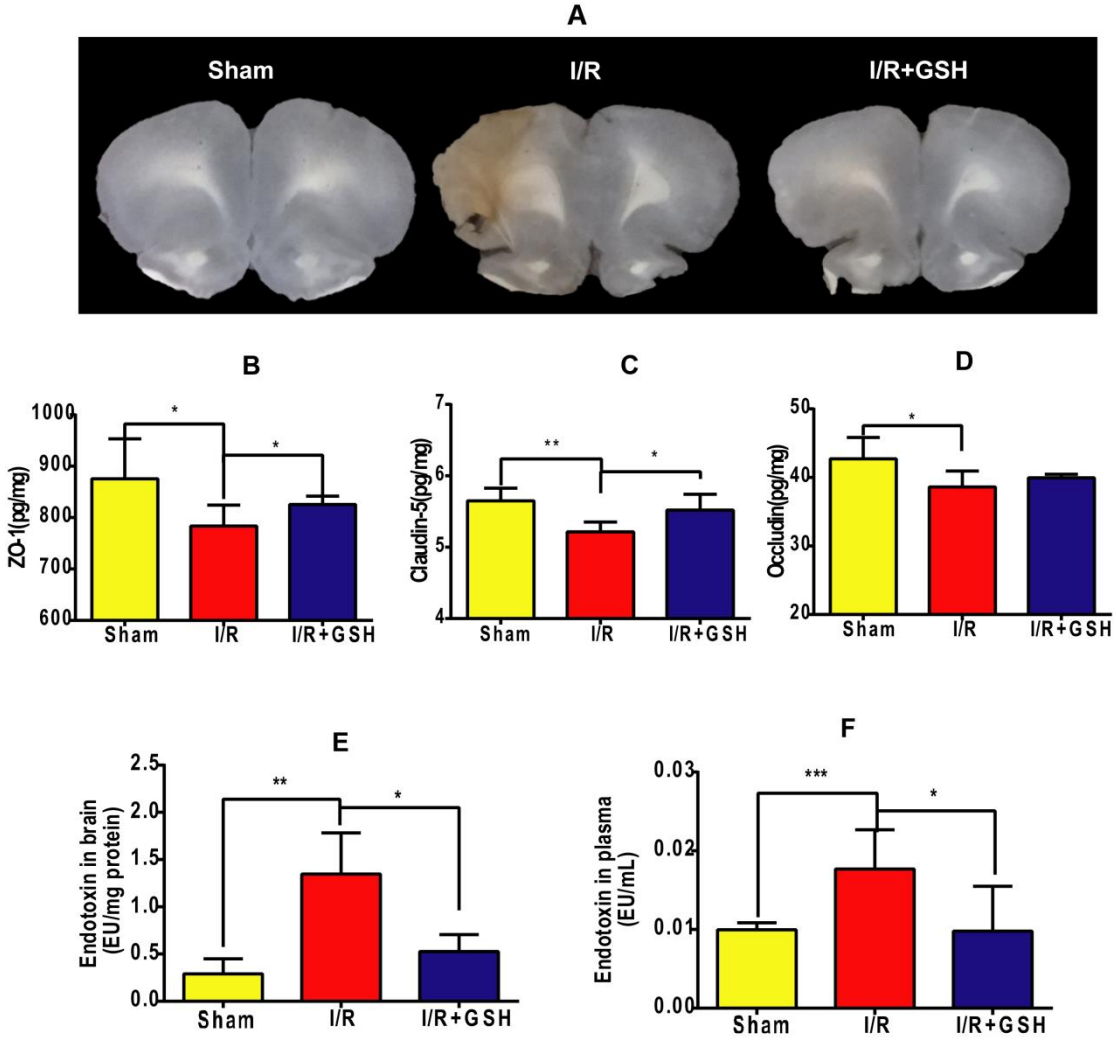

Figure 7

Supplemental Data to Drug metabolism and Disposition

Insights into the authentic active ingredients and action sites of oral exogenous glutathione in the treatment of ischemic brain injury based on pharmacokinetic-pharmacodynamic studies

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Table S1 Optimized MRM parameters of GSH, CYS, CYS-GLY, γ-GC, [$^{3\text{-}13}$C]GSH, [$^{3\text{-}13}$C]CYS-GLY and internal standard for LC-MS/MS determination.

Figure S1 Migration ratio of hCMEC/D3 at 12h (A) or 24h (B) after treating GSH or BSO. Data are mean ± SD. **P < 0.05, **P < 0.01 and **** P < 0.0001* by unpaired two-tailed Student's t-test (n=6).

Figure S2 The distribution of CYS in rat tissues after intragastrical administration of GSH at 250mg/kg. (A) Plasma (B) Heart, (C) Liver, (D) Kidney, (E) Stomach, (F) Duodenum, (G) Jejunum, (H) Ileum, (I) Colon.

Figure S3 The distribution of CYS-GLY in rat tissues after intragastrical administration of GSH at 250mg/kg. (A) Plasma (B) Heart, (C) Liver, (D) Kidney, (E) Stomach, (F) Duodenum, (G) Jejunum, (H) Ileum, (I) Colon.

Figure S4 The distribution of γ-GC in rat tissues after intragastrical administration of GSH at 250mg/kg. (A) Plasma (B) Heart, (C) Liver, (D) Kidney, (E) Stomach, (F) Duodenum, (G) Jejunum, (H) Ileum, (I) Colon.

Figure S5 The distribution of Glu in rat tissues after intragastrical administration of GSH at 250mg/kg. (A) Plasma (B) Heart, (C) Liver, (D) Kidney, (E) Stomach, (F) Duodenum, (G) Jejunum, (H) Ileum, (I) Colon.

Figure S6 The distribution of GLY in rat tissues after intragastrical administration of GSH at 250mg/kg. (A) Plasma (B) Heart, (C) Liver, (D) Kidney, (E) Stomach, (F) Duodenum, (G) Jejunum, (H) Ileum, (I) Colon.

Figure S7 The mRNA relative expression of GCLc in the rat liver. Data are mean ± SD. ***P < 0.001 by unpaired two-tailed Student's t-test.

Table S1 Optimized MRM parameters of GSH, CYS, CYS-GLY, γ-GC, [$^{3\text{-}13}$C]GSH, [$^{3\text{-}13}$C]CYS-GLY and internal standard for LC-MS/MS determination.

Analytes	Precursor (*m/z*)	Product (*m/z*)	DP (V)	CE (V)
GSH	433.1	304.1	40	21
CYS	246.9	158.2	55	30
CYS-GLY	304	287.1	50	18
γ-GC	376	247	81	20
[$^{3\text{-}13}$C]-L-GSH	436.7	307.1	40	21
[$^{3\text{-}13}$C]CYS-GLY	307.1	290.1	82	18
CAP(IS)	343.1	228.1	50	22

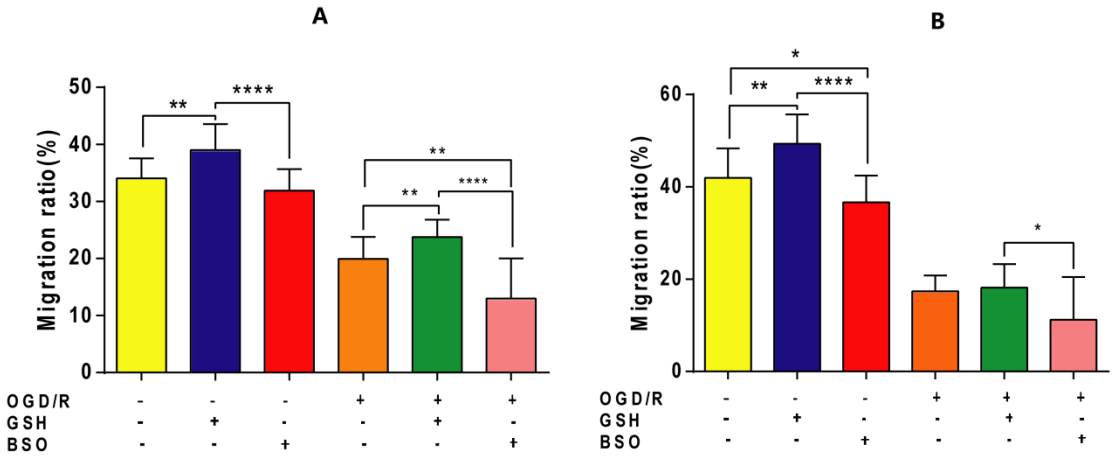


Figure S1 Migration ratio of hCMEC/D3 at 12 h (A) or 24 h (B) after treating GSH or BSO. Data are mean ± SD. **P < 0.05, **P < 0.01 and **** P < 0.0001* by unpaired two-tailed Student's t-test (n=6).

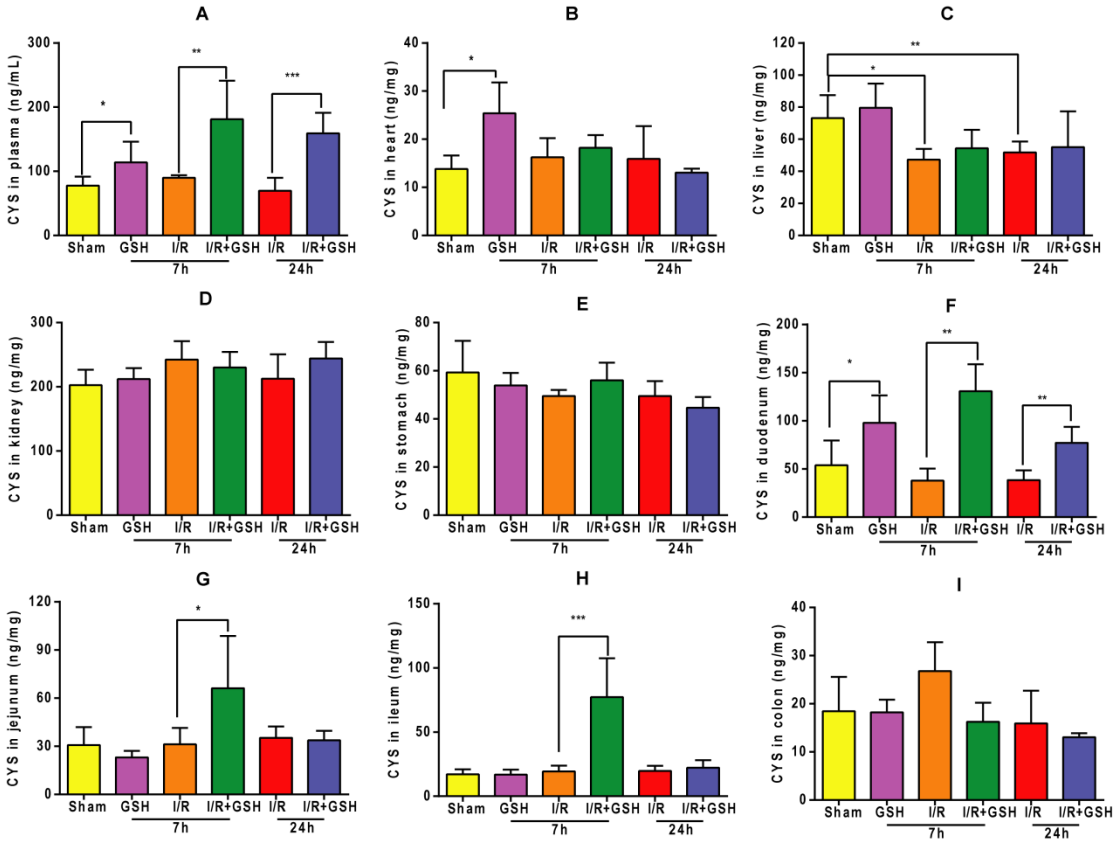


Figure S2 The distribution of CYS in rat tissues after intragastrical administration of GSH at 250mg/kg. (A) Plasma, (B) Heart, (C) Liver, (D) Kidney, (E) Stomach, (F) Duodenum, (G) Jejunum, (H) Ileum, (I) Colon.

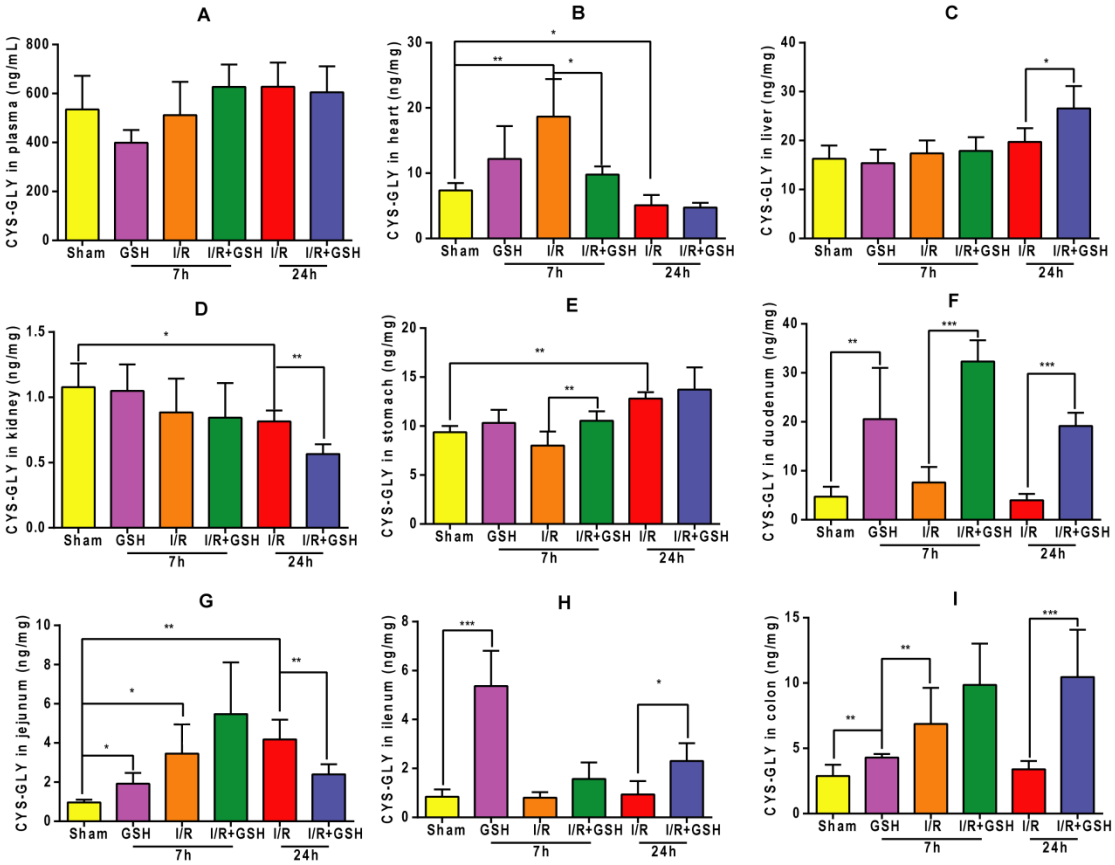


Figure S3 The distribution of CYS-GLY in rat tissues after intragastrical administration of GSH at 250mg/kg. (A) Plasma, (B) Heart, (C) Liver, (D) Kidney, (E) Stomach, (F) Duodenum, (G) Jejunum, (H) Ileum, (I) Colon.

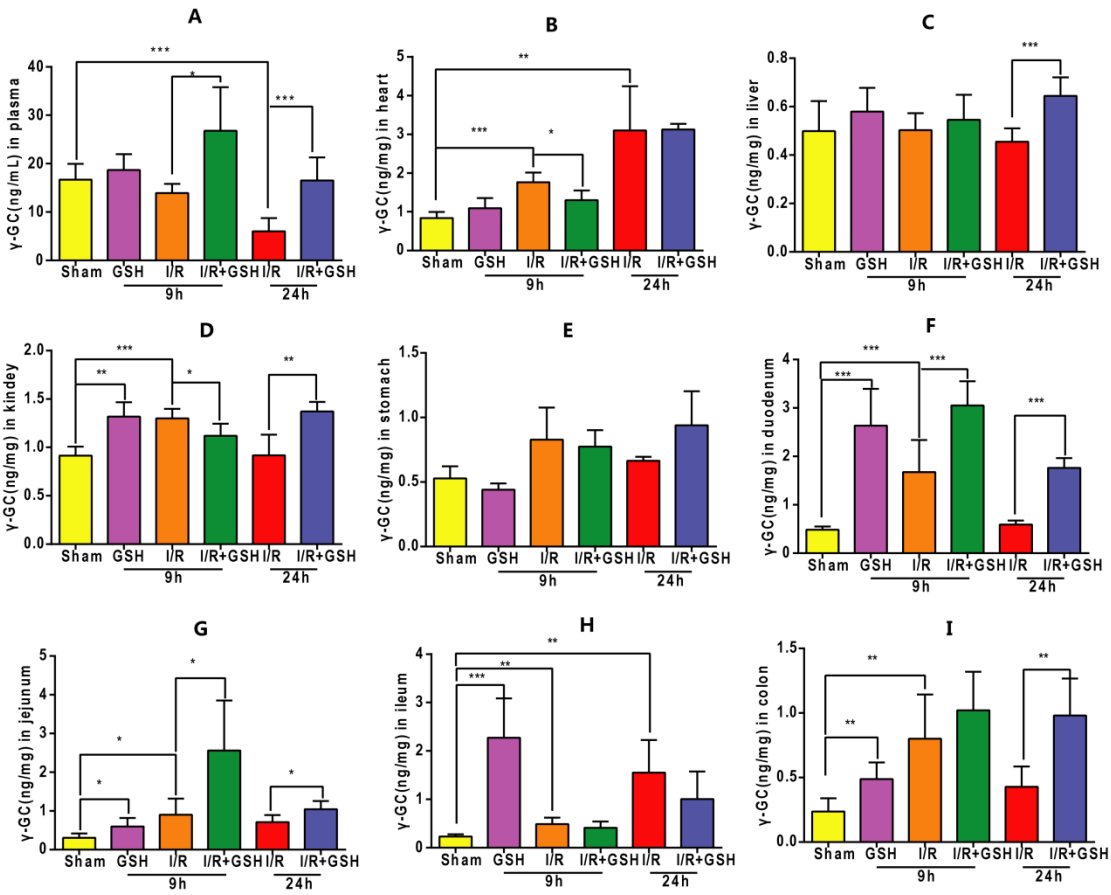


Figure S4 The distribution of γ-GC in rat tissues after intragastrical administration of GSH at 250mg/kg. (A) Plasma, (B) Heart, (C) Liver, (D) Kidney, (E) Stomach, (F) Duodenum, (G) Jejunum, (H) Ileum, (I) Colon.

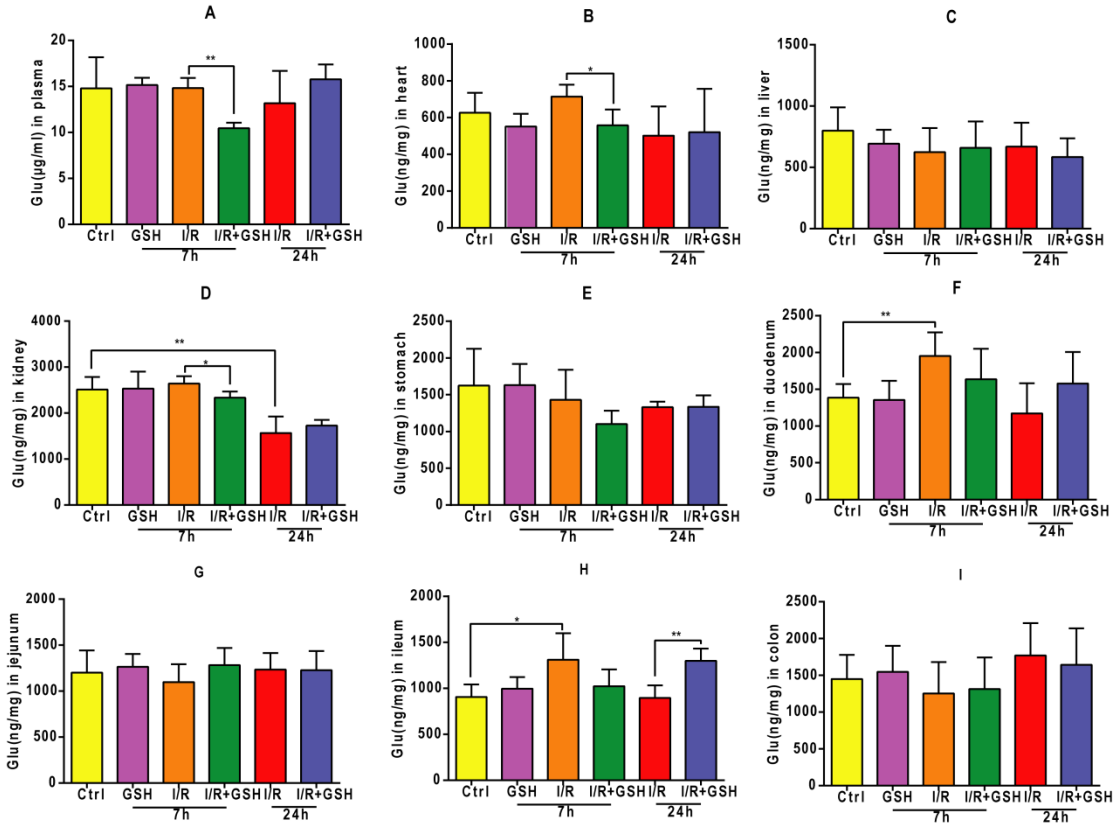


Figure S5 The distribution of Glu in rat tissues after intragastrical administration of GSH at 250mg/kg. (A) Plasma, (B) Heart, (C) Liver, (D) Kidney, (E) Stomach, (F) Duodenum, (G) Jejunum, (H) Ileum, (I) Colon.

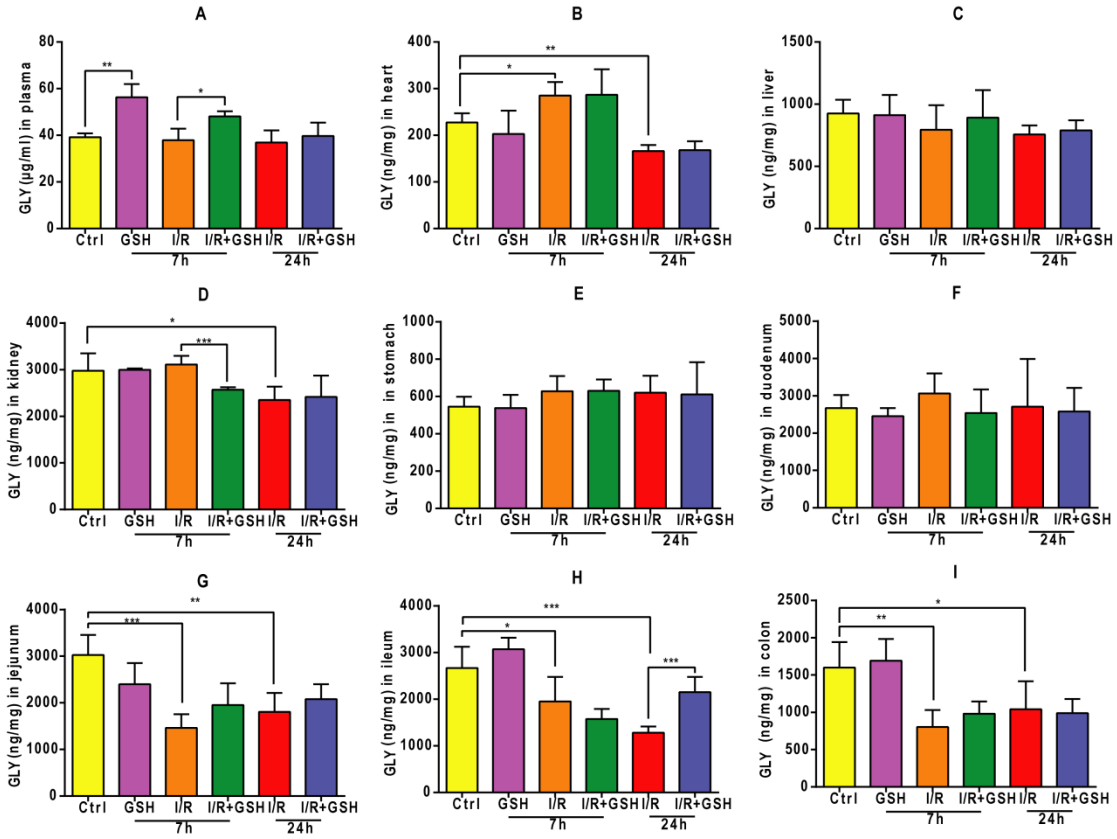
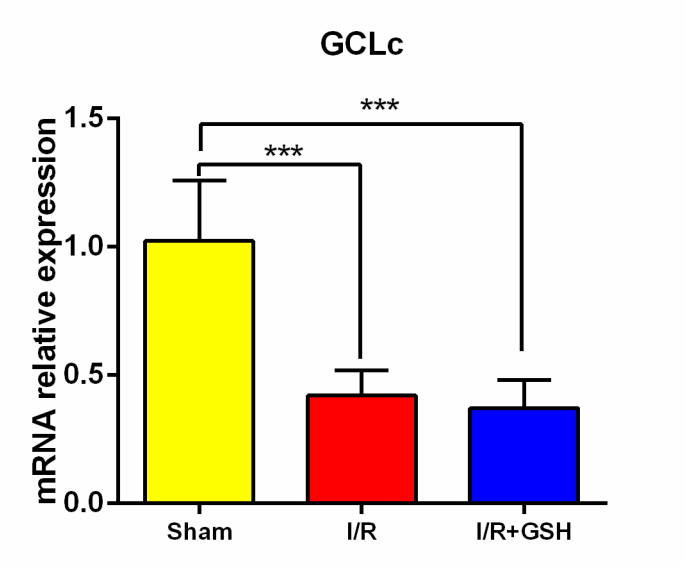


Figure S6 The distribution of GLY in rat tissues after intragastrical administration of GSH at 250mg/kg. (A) Plasma, (B) Heart, (C) Liver, (D) Kidney, (E) Stomach, (F) Duodenum, (G) Jejunum, (H) Ileum, (I) Colon.



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