ORIGINAL RESEARCH

Hydroxysafflor Yellow A Exerts Neuroprotective Effects by Inhibiting Protein Carbonyl Formation in Cerebral Ischemia-Reperfusion Injury

Ruijie Zhao, MM; Kun Wang, MM; Xi Zhang, MM; Yang Zhao, MM; Xipeng Li, MM

ABSTRACT

Protein carbonylation by reactive oxygen species (ROS) is an important factor in the pathogenesis of cerebral ischemia-reperfusion injury (CIRI). Carbonyls are mainly produced by peroxynitrite (ONOO-) and hemin/hydrogen peroxide (H_2O_2)/sodium nitrite ($NaNO_2$)-mediated reactions. As the main active water-soluble chalcone chemical ingredient derived from *Carthamus tinctorius L*, hydroxysafflor yellow A (HSYA) has been increasingly applied in the treatment of cerebrovascular disease (CVD).

In this study, rats were randomly divided into 3 groups: the sham-surgery group (sham), the CIRI group (CIRI) and the CIRI treated with HSYA group (HSYA). We evaluated the protective properties of HSYA in a CIRI model *in vivo*, assessed its efficacy against $ONOO^-$ and hemin/H_2O_2/$NaNO_2$-induced oxidative damage to cerebral cortical tissues *in vitro*, and explored the probable molecular mechanisms underlying its neuroprotective effects. The results showed that HSYA protected rats against CIRI by improving their neurological function score ($P < .05$), reducing infarct volume ($P < .01$), decreasing the content of protein carbonyls ($P < .01$) and elevating the glutathione (GSH) levels ($P < .01$). Further *in vitro* investigations found that HSYA pretreatment could inhibit protein carbonylation induced by exogenous $ONOO^-$ application in cortical brain tissues in a dose-dependent manner ($P < .01$). In terms of hemin/H_2O_2/$NaNO_2$-triggered oxidative damage, HSYA slightly promoted the formation of carbonyl groups ($P < .05$).

In conclusion, this study demonstrates that the neuroprotective capabilities of HSYA in CIRI are attributable, at least in part, to the enhancement in antioxidant capacity and the attenuation of protein oxidation, probably via the combined processes of $ONOO^-$ scavenging and the suppression of protein carbonyl formation. (*Altern Ther Health Med.* [E-pub ahead of print.])

Ruijie Zhao, MM; **Xi Zhang**, MM; **Yang Zhao**, MM; **Xipeng Li**, MM, Department of Neurology; Xingtai People's Hospital; Hebei Medical University Affiliated Hospital; Xingtai, Hebei Province; China. **Kun Wang**, MM, Department of Neurology; Xingtai Third Hospital; Xingtai, Hebei Province; China.

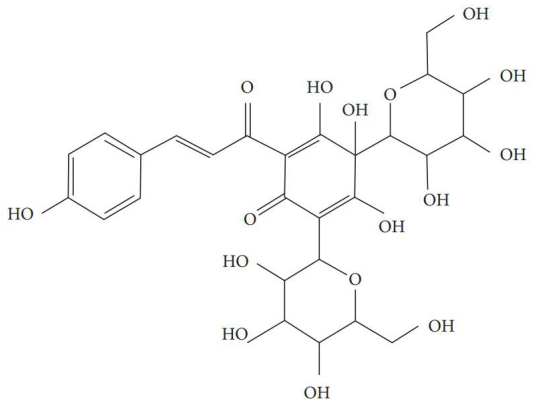
Corresponding author: Xipeng Li, MM
E-mail: lxp731221@163.com

INTRODUCTION

In patients with acute ischemic stroke, cerebral ischemia-reperfusion injury (CIRI) is an extremely complex pathophysiological process that can aggravate the pathological damage to ischemic brain tissues and worsen patient conditions.[1] Oxidative stress is one of the most important pathophysiological mechanisms of CIRI during the process of revascularization therapy, and plays a core role in the ischemic cascade reactions that eventually cause more severe brain damage after reperfusion.[2] When the generation of highly reactive molecules, such as reactive nitrogen species (RNS) and reactive oxygen species (ROS), exceeds the scavenging capability of antioxidant response systems in the body, the dynamic balance of the biological systems is disrupted and oxidative stress occurs. This results in cell death through protein oxidation, modifications to nucleic acids and lipid peroxidation.[3-5] At present, the most reliable biomarker of ROS-mediated protein oxidation, which changes protein structure and function, is the formation of carbonyl groups in proteins.[6-8] Furthermore, tests based on the presence of carbonyl groups have several advantages compared with tests that rely on the monitoring of lipid peroxidation under oxidative stress conditions, such as the detectability of early changes and relatively stable properties.[9]

Over the years, the pivotal routes *in vivo* found in the literature for protein oxidative/nitrative modification are hemin/hydrogen peroxide (H_2O_2)/sodium nitrite ($NaNO_2^-$) and peroxynitrite ($ONOO^-$)-mediated pathways involving free radicals and iron catalysis.[10-12] We have also come to realize that antioxidants can potently decrease the effects of

Figure 1. The structure of hydroxysafflor yellow A.



oxidative attack on proteins regardless of the mechanism of oxidative modification. However, various antioxidants may have different effects on $ONOO^-$- or hemin/H_2O_2/$NaNO_2$-triggered protein carbonylation, which is worthy of further study and exploration.

With the continuous development of traditional Chinese medicine (TCM) in recent years, Chinese herbal medicines and their derivatives have been increasingly applied in clinical practice, and they have shown great research value and potential in the treatment of CVD. Safflower (*Carthamus tinctorius L*), a valuable TCM used to treat the symptoms of blood stasis and promote blood circulation, has a pungent taste, enters the heart and liver meridians and contains a variety of pharmacological components. Hydroxysafflor yellow A (HSYA) is a natural single chalcone glycoside compound extracted from safflower (Figure 1). It is the monomer water-soluble ingredient with the most effective pharmacological action.[13] Previous studies have shown that HSYA has anti-inflammatory,[14,15] anti-apoptotic,[16] antioxidative[14,17] and anti-atherosclerotic properties.[18] Moreover, a recent study demonstrated that HSYA treatment significantly inhibits the opening of mitochondrial permeability transition pores, as well as decreases ROS generation in brain mitochondria after CIRI.[19] However, the detailed molecular basis underlying the biological properties of HSYA has not been properly described.

In this study, we assessed whether HSYA protected brain tissue proteins from oxidative impairment caused by CIRI through the inhibition of protein carbonylation *in vivo*. Furthermore, we measured glutathione (GSH) levels to evaluate the salutary effects of HSYA on CIRI. In addition, we investigated the mechanism underlying the antioxidative properties of HSYA. To achieve these aims, the effect of HSYA on carbonylated proteins was tested when cerebral cortical proteins were exposed to $ONOO^-$ or hemin/H_2O_2/$NaNO_2$ *in vitro*.

MATERIALS AND METHODS

Chemicals and Reagents

$ONOO^-$ was obtained from Millipore (Temecula, California, USA). Hemin, 2,3,5-triphenyltetrazolium chloride (TTC), dimethyl sulfoxide (DMSO), and 2,4-dinitrophenylhydrazine (DNPH) were provided by Sigma-Aldrich (St. Louis, Missouri, USA). Guanidine hydrochloride was purchased from Amresco, Inc. (Solon, Ohio, USA). HSYA (purity > 98%) was obtained from Zhejiang Yongning Pharmaceutical Co., Ltd. (Zhejiang, China). Phosphate-buffered saline (PBS) and phenylmethylsulfonyl fluoride (PMSF) were provided by Solarbio (Beijing, China). Reduced glutathione (GSH) assay kit was provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals used were of the highest commercially available analytical grade.

Animals

The Academy of Military Medical Sciences (Beijing, China) provided pathogen-free male Sprague-Dawley (SD) rats weighing 280 to 320 g. A total of 15 rats were randomly divided into the sham-surgery group (sham), the CIRI group (CIRI) and the CIRI treated with HSYA group (HSYA), with 5 rats in each group. The rats were housed in cages and given sufficient water and food with no restrictions in a room with a temperature of 22°C ± 2°C during the experiment. Adaptation was allowed for at least 7 days before experimentation. Normal rats were fasted for 12 hours and permitted to drink freely ahead of treatment. The National Institutes of Health *Guide for the Care and Use of Laboratory Animals* was followed for all animal experiments.

The research protocol was reviewed and approved by the ethics committee of Xingtai People's Hospital in China.

Establishment of CIRI Model and Treatment

As described in a 2012 study by Sun, et al,[20] intraluminal middle cerebral artery occlusion (MCAO) was used to establish a CIRI model. Briefly, following anesthetization with an intraperitoneal injection of 10% chloral hydrate (3.5 mL/kg), the right carotid artery was fully exposed and bluntly dissected. Through a minor V-shaped incision in the external carotid artery, the suture with a heat-rounded tip was carefully and slowly inserted into the internal carotid artery until it reached the beginning of the middle cerebral artery when encountering slight resistance. After 1 hour of MCAO, the suture was pulled back for 24 hours to achieve reperfusion.

A similar surgical procedure but without MCAO was performed in the rats in the sham-surgery group. A total of 15 rats were randomly divided into 3 groups: the sham-surgery group (sham), the CIRI group (CIRI), and the CIRI treated with HSYA group (HSYA). HSYA (10 mg/kg) was administered via caudal vein injection half an hour before MCAO. The rats in both the CIRI and sham groups received an equivalent volume of saline.

Evaluation of Neurological Deficits

Evaluation of neurological deficits was carried out by determining modified neurological Severity Scores (mNSS) 24 hours after MCAO, including balance, sensory, motor and reflex tests. Neurological function scores ranged from 0 to 18 (minimal deficiency, 0; maximum deficiency, 18).[21]

Infarct Volume Calculation

In order to determine the volume of the brain infarct 24 hours after surgery, TTC staining was carried out. The rat brains were rapidly extracted and sliced into 2 mm-thick coronal sections. The brain tissue slices were fixed overnight using 4% paraformaldehyde at 4°C after having been treated with 2% TTC for 20 minutes at 37°C. Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, Maryland USA) was used to determine the total hemisphere area and the infarcted area of each coronal slice. The percentage area of the ischemic hemisphere was used to calculate cerebral infarct volume.

Tissue Preparation

SD rats were euthanized by decapitation under anesthesia with 10% chloral hydrate. The cerebral cortex was quickly dissected on ice, the tissue was rinsed with saline at 4°C, and the arachnoid membrane was carefully removed. The tissues were homogenized at 4°C in a mixture of PMSF/PBS (1:100 v/v) and subsequently centrifuged at 4°C at 13 000 rpm for 15 minutes. Finally, the supernatant of the brain homogenates was collected and its protein concentration was determined using the Lowry method.

Sample Treatment

A total of 20 normal SD rats were allocated at random into 4 groups (5 rats per group): blank, control, low-dose HSYA (HSYA-L, 0.1 mM) and high-dose HSYA (HSYA-H, 1 mM). All groups were treated with 50 mM phosphate-buffered saline (PBS) solution and normal rat cortical brain tissue homogenate in which the final protein concentration was 4 mg/mL, while the control group was treated with $ONOO^-$ (5 mM) or a mixture of hemin (100 μM)/H_2O_2 (1 mM)/$NaNO_2$ (10 mM) for 30 minutes at 37°C. On the basis of the control group, the treatment group was pre-incubated with different doses of HSYA (HSYA-L, 0.1 mM; HSYA-H, 1 mM) for 5 minutes at 37°C. Finally, the reaction was terminated on ice, and the final reaction mixtures were utilized in the subsequent tests.

GSH Determination

GSH content in the cerebral cortex was detected using 5, 5'-dithiobis-(2-nitrobenzoic acid). Briefly, the GSH assay kit's working solution was prepared based on the manufacturer's directions and mixed with the supernatant of the cerebral cortex. The absorbance of the mixture was estimated at 405 nm. GSH content in the supernatant was determined relative to the standard absorbance.

Carbonyl Formation Assay

The production of 2,4-DNPH derivatives was employed to examine carbonyl groups in proteins by spectrophotometry, which accurately reflects protein oxidation. The method utilized for the analysis of protein carbonyls was detailed in our previous paper.[20] In brief, streptomycin sulfate solution (10% weight/volume [w/v]) was added to the brain homogenate and fully mixed. The mixture was then centrifuged for 30 minutes at 11 000 g to precipitate the DNA, and the resulting supernatant was evenly mixed with 10 mM 2,4-DNPH (in 2 M hydrochloric acid [HCl]) and fully shaken once every 10 minutes for 2 hours in dark conditions. Then, trichloroacetic acid (10% final concentration) was added, and the mixture was centrifuged to obtain the precipitate. Ethylacetate and ethanol (1:1) were added 3 times to wash free dinitrophenylhydrazine (DNPH). The final precipitate was dissolved in 6 M guanidine hydrochloride. Protein concentration was determined based on the absorbance of the HCl sample at 280 nm. Protein carbonyls were detected by measuring the absorbance of the DNPH sample at 370 nm. Results were expressed as nmol/mg.

Statistical analyses

Data were represented as mean ± standard deviation (SD). Data analysis was performed using the IBM SPSS version 25.0 software (IBM, Armonk, New York USA). One-way ANOVA was used for multiple group comparisons and the least significant difference (LSD) test was used for post-hoc comparisons. The *t* test was employed to make comparisons between the 2 groups. $P < .05$ was deemed statistically significant.

RESULTS

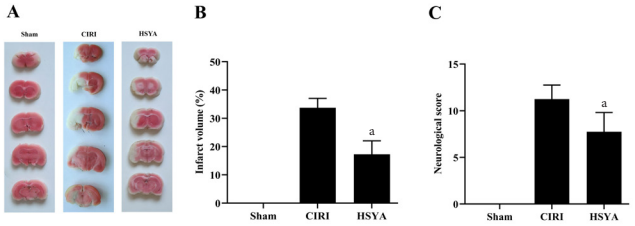
HSYA Pretreatment Reduced Infarct Volume and Enhanced Neurological Function in Rats with CIRI

Infarct volume and neurological function were assessed in rats with CIRI to test the neuroprotection effects of HSYA. As shown in Figures 2A and 2B, TTC staining revealed that all brain slices in the sham group were stained red, whereas the frontal lobe, temporal lobe, parietal lobe cortex and striatum in the CIRI group appeared white, indicating the presence of an infarct. At 24 hours after MCAO, CIRI caused significant cerebral infarction, and HSYA pretreatment significantly decreased infarct volume (33.75 ± 3.30% vs 17.25 ± 4.79%; $P < .01$; Figure 2B). The rats in the sham group did not show any neurological impairments (Figure 2C). Compared with the sham group, modified neurological severity scores were obviously enhanced in the CIRI group. The group pretreated with 10 mg/kg HSYA had markedly lower scores than the CIRI group (7.75 ± 2.06 vs 1.25 ± 1.50; $P < .05$). HSYA appears to protect against CIRI based on changes in neurological function and cerebral infarct volume.

HSYA Reduces CIRI-Induced Protein Oxidation and Enhances the Antioxidant Capacity of Brain Tissue

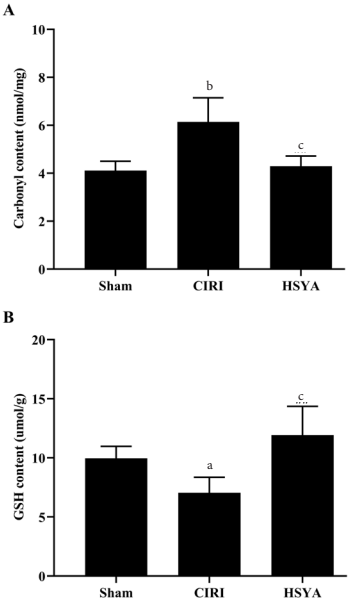
We measured protein carbonyls *in vivo* to investigate the impact of HSYA on cerebral protein oxidation. Carbonylation was found to be higher in the brain tissue of rats with CIRI compared with the sham group (6.14 ± 1.01 nmol/mg vs 4.11 ± 0.39 nmol/mg; $P < .01$; Figure 3A), showing that CIRI caused increased ROS production and protein oxidization. The CIRI-induced carbonyl content was dramatically reduced by HSYA pretreatment (10 mg/kg) (6.14 ± 1.01 nmol/mg vs 4.29 ± 0.43 nmol/mg; $P < .01$; Figure 3A), suggesting that HSYA can prevent the carbonylation of proteins resulting from CIRI.

Figure 2. Effects of HSYA on the neurological deficit and cerebral infarct volume following CIRI in rats. (**2A**) Images of TTC-stained brain slices, which depict 1 representative cerebral tissue from each test group. The infarct area of coronal brain slices was stainless, whereas the non-infarct area was stained red. (**2B**) Percentage analysis of the cerebral infarct volume. The bar graph shows the infarct volume as a percentage of the ischemic hemisphere's area. (**2C**) Neurological deficit scores analysis. HSYA (10 mg/kg) was given via caudal vein injection half an hour before 1 hour of ischemia followed by 24 hours of reperfusion. The CIRI and sham rat groups were given the same amount of saline. Data are presented as mean ± SD. n = 5



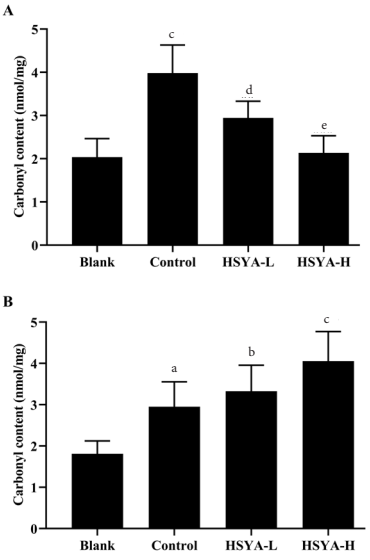
[a]$P < .01$ vs CIRI group.

Figure 3. Effects of HSYA on protein carbonylation and antioxidant capacity following CIRI in rats *in vivo*. HSYA (10 mg/kg) was given via caudal vein injection half an hour before 1 hour of ischemia followed by 24 hours of reperfusion. The CIRI and sham rat groups were given the same amount of saline. (**3A**) Effect of HSYA on protein carbonylation following CIRI in rats *in vivo*. The production of carbonyl groups in proteins was used to assess protein carbonylation. (**3B**) Effect of HSYA on antioxidant capacity following CIRI in rats *in vivo*. Antioxidant capacity was detected by the GSH content. Data are presented as mean ± SD, n=5



[a]$P < .05$
[b]$P < .01$ vs sham
[c]$P < .01$ vs CIRI

Figure 4. Effects of HSYA on protein carbonylation *in vitro*. (**4A**) Effect of HSYA on $ONOO^-$-induced protein carbonylation *in vitro*. (**4B**) Effect of HSYA on protein carbonylation mediated by hemin/H_2O_2/$NaNO_2$ *in vitro*. The production of carbonyl groups in proteins was used to assess protein carbonylation. The data are shown as mean ± SD. n =5



[a]$P < .05$
[b]$P < .01$
[c]$P < .001$, vs blank
[d]$P < .01$
[e]$P < .001$ vs control.

GSH, the primary indicator of antioxidant capability, is crucial for preserving the physiological processes and redox state homeostasis of all cells *in vivo*.[22] We measured the GSH levels to test the antioxidant capacity of HSYA. As indicated in Figure 3B, the GSH content in the brains of rats with CIRI was significantly lower compared with the sham group (7.04 ± 1.31 μmol/g vs 9.96 ± 1.02 μmol/g; $P < .05$). Compared with the CIRI group, the GSH content in the brains of rats from the HSYA group (11.93 ± 2.43 μmol/g) increased significantly (by 69.46%) when HSYA (10 mg/kg) was administered half an hour before MCAO ($P < .01$).

HSYA Inhibits Protein Carbonylation Induced by $ONOO^-$

As the major marker of oxidative protein damage, a small amount of carbonyl protein (2.04 ± 0.43 nmol/mg) in the cortical brain homogenate of normal rats was monitored using DNPH. Exposure to $ONOO^-$ resulted in increased protein carbonyls (3.98 ± 0.65 nmol/mg). However, in the presence of HSYA, the elevated protein carbonyl levels were significantly reduced in a concentration-dependent manner (2.94 ± 0.39 nmol/mg and 2.14 ± 0.40 nmol/mg for the HSYA-L and HSYA-H groups; $P < .01$, respectively; Figure 4A). HSYA pretreatment significantly protected against oxidative damage *in vitro*, irrespective of iron chelation, as demonstrated by its interaction with free radicals derived from $ONOO^-$.

HSYA Enhances Hemin-mediated Protein Carbonylation

As shown in Figure 4B, when the rat brains were treated with hemin (100μM)/H_2O_2 (1mM)/$NaNO_2$ (10mM), carbonyl groups were significantly increased (2.95 ± 0.60 nmol/mg vs 1.81 ± 0.31 nmol/mg in the blank group; $P < .05$), demonstrating that there was an excessive amount of ROS being produced. Contrary to its function in $ONOO^-$-triggered protein carbonylation, the concentration-dependent decreasing trend of protein carbonylation was not seen in the administration doses (0.1 and 1 mM) of HSYA (3.26 ± 0.62 nmol/mg and 3.66 ± 0.55 nmol/mg for the HSYA-L and the HSYA-H groups, respectively). HSYA did not exhibit a protective effect on hemin-mediated oxidative injury to proteins but exerted pro-oxidative properties *in vitro* at the concentrations used in this study (Figure 4B).

DISCUSSION

In this study, we demonstrated that HSYA pretreatment notably decreased infarct volume, improved neurological function and upregulated the expression of GSH in a rat model of CIRI. We established 2 widely recognized oxidative models (hemin/H_2O_2/$NaNO_2$ and $ONOO^-$ exposure) *in vitro*, and our data demonstrated that HSYA can inhibit $ONOO^-$-mediated protein carbonylation in a concentration-dependent manner. However, HSYA exerted slight pro-oxidative properties at the concentrations used in this study when cerebral cortical proteins were exposed to hemin/H_2O_2/$NaNO_2$ *in vitro*. Therefore, our study implies that HSYA may be an effective, viable therapeutic agent for ischemic stroke treatment, as well as a potential antioxidant supplement for preventive treatment in populations considered to be at high-risk for ischemic stroke.

Protein oxidation is a major post-translational alteration of proteins by ROS. As reported in the literature, protein oxidative alteration is involved in the pathogenesis of many human disorders, such as atherosclerosis, cardiovascular disease, cancer, diabetes and aging.[23,24] Protein oxidation has been confirmed to be closely associated with cerebral ischemic damage as well as neurodegenerative diseases, and increased protein carbonyl levels have been detected in patients with stroke, whereas decreased carbonyl group content has been reported in patients after rehabilitation.[25] In this study, we also found that the carbonyl group content in the brain of the CIRI group was much higher than in the sham group, implying that the excessive generation of ROS resulting from CIRI leads to oxidative modification of brain proteins.

Protein carbonylation is an irreversible post-translational modification of proteins that is not catalyzed by enzymes, and is considered to be the most common indicator of protein oxidation.[26,27] Protein carbonylation is defined as the process by which ROS attacks the side chains of amino acids, resulting in the formation of carbonyl derivates.[26,28] On one hand, ROS can directly create carbonyls on arginine, lysine and proline via metal-catalyzed oxidation and hypochlorous acid; on the other hand, lipid peroxidation could indirectly yield carbonyl modification through covalent adsorption of reactive carbonyl species to amino acids such as histidine, lysine and cysteine.[29] These carbonylation modifications can affect the structure, activity and function of proteins. For instance, oxidative stress can elicit inactivation of zonula occludens-1 by carbonyl modification and destruction of the permeability of the blood-brain barrier.[17] Thus, compounds capable of inhibiting protein carbonylation would presumably help protect protein function and exert neuroprotective properties. In fact, we observed attenuation of carbonyl modification, reduction of infarct volume, improvement of neurologic function and elevation of GSH in the group of rats treated with HSYA compared with the CIRI group. These results implied that anti-carbonylative action and enhancement in the antioxidant capacity play a significant role in the neuroprotective mechanisms of HSYA during CIRI.

Previous studies have reported that the primary routes of protein oxidative/nitrative modification that take place in the body include hemin/H_2O_2/$NaNO_2$- and $ONOO^-$-mediated processes involving iron catalysis and free radicals.[10-12] $ONOO^-$, a very active oxidant generated by the overproduction of nitric oxide and superoxide anions, may play a neurotoxic role via a variety of processes, including protein oxidation. As shown in this study, $ONOO^-$ promoted the production of protein carbonyls. However, $ONOO^-$ scavengers can mitigate the level of protein carbonylation in the rat brain.[30] To date, most studies have focused on the protective effects of antioxidants against $ONOO^-$-dependent pathways; little attention has been paid to hemin/H_2O_2/$NaNO_2$-induced reactions. Hemin is derived from the products of hemoglobin degradation and can catalyze protein oxidation, which eventually leads to the injury or death of brain cells, including neurons.[31] Hemin exerts cytotoxicity via iron, which is a major generator of ROS that can initiate a chain reaction of detrimental processes, resulting in cell death or injury.[32] On the other hand, hemin itself can take part in redox reactions, generating free radicals and triggering cytotoxic reactions.[33] H_2O_2 and $NaNO_2$ are essential for protein oxidation.[34] Furthermore, as a highly reactive ROS, H_2O_2 can react with transition metal ions such as Fe^{2+} or Cu^{2+}, generating an extremely active hydroxyl radical (•OH) by the Fenton reaction.[35] •OH is a potent free radical that reacts with proteins, lipids and nucleic acids.

In this study, we performed *in vitro* assessments of protein carbonylation to evaluate the antioxidative mechanism of HSYA. The results from our study clearly showed that the impact of HSYA on protein carbonylation via the 2 oxidative reaction systems differed markedly. HSYA was highly effective and able to prevent protein carbonylation induced by exogenous $ONOO^-$ application in cortical brain tissue in a dose-dependent manner. In contrast, in the case of hemin/H_2O_2/$NaNO_2$-triggered protein oxidation, HSYA pretreatment slightly increased the formation of carbonyl groups. We speculate that the addition of HSYA to the hemin/H_2O_2/$NaNO_2$ reaction system can form a novel intermediate that promotes protein oxidation. Generally, the higher the number of hydroxyl group substitutions, the more

powerful the pro-oxidant and antioxidant properties are.[11] Hence, we suppose that the inhibitory effect of HSYA on protein carbonylation applies mainly to the $ONOO^-$ pathway and derives from its ability to combine with $ONOO^-$ and/or its generated free radicals. To the best of our knowledge, the effects of HSYA on protein carbonylation *in vitro* via both the hemin/H_2O_2/$NaNO_2$ and $ONOO^-$ pathways were tested for the first time in our study.

Because of the deleterious effects of oxidation on proteins, previous studies have preliminarily recognized that oxidative stress is a potential therapeutic target, and the utilization of antioxidants is a viable therapeutic method for preventing oxidative stress involving iron and free radicals in ischemic stroke.[1,36] HSYA is a flavonoid in polyphenols that are characterized by having more than 1 phenolic hydroxyl group. Flavonoids are the most abundant polyphenols in nature and can exert anti-stroke effects by acting on multiple pathways and targets, in contrast to traditional medications.[37] Of more importance, flavonoids are also the main active ingredients of many Chinese herbal medicines, such as Baicalin, Epigallocatechin gallate, Quercetin, Myricetin, Isorhamnetin and Neohesperidin, which exert antioxidant activities by decreasing the level of ROS-mediated protein oxidation and increasing the content of antioxidants such as GSH and superoxide dismutase. The results of this study were in line with the above-mentioned previous findings.[36] Therefore, we speculate that the inhibition of protein oxidation in the brain by HSYA is related to the antioxidant activity conferred by the multiple phenolic hydroxyl groups in its structure.

In the body, GSH is not only the most abundant non-protein thiol but also the most essential non-enzymatic intracellular antioxidant.[22] GSH can directly act as an antioxidant via non-enzymatic reactions with ROS/RNS, including nitric oxide, superoxide, singlet oxygen, $^{\bullet}OH$, H_2O_2 and $ONOO^-$. It can also indirectly serve as an enzyme cofactor for GSH peroxidase to degrade H_2O_2 and hydroperoxides and is involved in detoxifying electrophilic xenobiotics via GSH-S-transferase.[22,38] Because of these protective functions, GSH is important not just in the maintenance of redox homeostasis in neurons, but also in the antioxidant defense system of neurons. Of note, it has been reported that GSH expression is upregulated during the recovery of neurological function after CIRI.[39] The results of our research revealed a marked decrease in GSH levels in the CIRI group, indicating that the antioxidant defenses were weakened after CIRI. However, HSYA pretreatment caused a significant increase in GSH levels, suggesting that HSYA could improve the antioxidant defense ability of the body by upregulating GSH expression, thereby exerting neuroprotective effects.

Study Limitations

However, this study has limitations. First of all, the sample size was small, due to the limitation of experimental materials and funds; there were only 5 rats in each group in this experiment. Although the effect of HSYA can be reflected to a certain extent, the sample size needs to be increased for the accuracy of scientific research and clinical promotion. Second, there was no positive control group in this study. If there were a positive drug control, it is more likely to illustrate the effectiveness of HSYA therapy. In addition, the deeper mechanism of action, such as molecular mechanism, gene regulation, pathway effect, etc., was not covered in this paper. In the follow-up study, we will create better experimental conditions, obtain enough sample size, select one or more of the currently recognized drugs, set up the relevant positive drug control group, and deeply explore the relevant mechanisms, establish the cell model *in vivo*, and carry out further research on the potential molecular mechanism of HSYA's therapeutic effect at the cellular level.

CONCLUSION

In conclusion, our data elucidated the neuroprotection effect of HSYA pretreatment on brain tissues induced by CIRI. The neuroprotective capabilities of HSYA injected half an hour before MCAO are attributable, at least partly, to the enhancement in antioxidant capacity and the attenuation in protein oxidation, probably via the combined processes of $ONOO^-$ scavenging and the suppression of protein carbonyl formation. Therefore, our study indicated that HSYA could be an effective, viable therapeutic agent for ischemic stroke treatment, as well as a potentially useful antioxidant supplement for the preventive treatment of populations considered to be at high risk for ischemic stroke.

AUTHOR CONTRIBUTIONS

Ruijie Zhaov and Kun Wang contributed equally to this paper and should be regarded as co-first authors.

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