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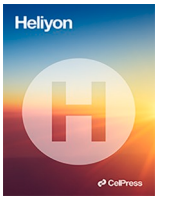

Research article

Aquaporins alteration revealed kidney damages in cerebral ischemia/reperfusion rats

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A R T I C L E I N F O

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Aqp4

A B S T R A C T

Background: Restoration of blood supply is a desired goal for the treatment of acute ischemic stroke. However, the restoration often leads to cerebral ischemia-reperfusion injury (CIR/I), which greatly increases the risk of non-neural organ damage. In particular, the acute kidney injury might be one of the most common complications.
Aims: The study aimed to understand the damage occurred and the potential molecular mechanisms.
Methods: The study was explored on the CIR/I rats generated by performing middle cerebral artery occlusion/reperfusion (MCAO/Reperfusion). The rats were evaluated with injury on the brains, followed by the non-neural organs including kidneys, livers, colons and stomachs. They were examined further with histopathological changes, and gene expression alterations by using RT-qPCR of ten aquaporins (*Aqps*) subtypes including *Aqp1 ~ Aqp9* and *Aqp11*. Furthermore, the *Aqps* expression profiles were constructed for each organ and analyzed by performing Principle Component Analysis. In addition, immunohistochemistry was explored to look at the protein expression of *Aqp1*, *Aqp2*, *Aqp3* and *Aqp4* in the rat kidneys.
Results: There was a prominent down-regulation profile in the MCAO/Reperfusion rat kidneys. The protein expression of Aqp1, Aqp2, Aqp3 and Aqp4 was decreased in the kidneys of the MCAO/Reperfusion rats. We suggested that the kidney was in the highest risk to be damaged following the CIR/I. Down-regulation of Aqp2, Aqp3 and Aqp4 was involved in the acute kidney injury induced by the CIR/I.

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1. Introduction

Ischemic stroke is the most frequent subtype of stroke and is the leading cause of disability and death in the world [1]. Restoration of the brain blood supply, named as "reperfusion", is a desired goal for treatment on acute ischemic stroke patients. However, when the blood supply of ischemic brain tissues is restored, cerebral ischemia-reperfusion injury (CIR/I) often occurs, worsening the brain damage [2,3]. Meanwhile, non-neural organs may be damaged within the first week after the stroke, adversely affecting stroke treatment [4,5]. In particular, as one of the common complications, acute renal injury (AKI) is known to be associated with poor prognostics, prolonged hospitalization and higher mortality [6]. In a clinical study of 45 consecutive stroke patients treated with the intravenous recombinant tissue plasminogen activator, the AKI incidence was 35.5 % and the in-hospital mortality was 50.0 % [7]. It is, therefore, necessary to understand the mechanism of AKI following the CIR/I.

The kidney, a central organ, has a function to maintain human body water homeostasis [8]. Aquaporins (AQPs) belong to membrane channel proteins discovered from bacteria to humans. In humans, there are thirteen AQPs subtypes (AQP0-AQP12). They are characterized into three sub-classes: AQP0 ~ AQP2, AQP4 ~ AQP6 and AQP8 are involved in orthodox AQPs; AQP3, AQP7, AQP9 and AQP10 belong to aquaglyceroporins; AQP11 and AQP12 are enrolled in super/unorthodox AQPs. They are widely expressed in human organs including brain, kidneys, colon, stomach, and liver. No single aquaporin isoform could be individually expressed at a single site [9]. Based on pore selectivity, the AQPs family could monitor transmembrane diffusion of water as well as various small molecules such as urea, CO_2, ammonia, H_2O_2, boric acid, glycerol and silicic acid [10]. In kidneys, multiple AQPs are expressed in different levels along the renal collecting ducts and tubules, and play key roles to control the fluid osmolality and electrolyte concentrations, to maintain acid-base balance and to remove toxins [8]. Particularly, AQP1, AQP2, AQP3 and AQP4 are known to be predominant and play important roles in the reabsorption of water and some solutes in the kidneys. Their alterations represent the response to the changes in the physiological intracellular and extracellular environment, and indicate the disturbance of the small molecule and water homeostasis involved in the organs [11,12]. Thus, overall AQP alterations shown in the kidneys would gain an insight into the influence on the CIR/I.

Aiming to understand the damage occurred in the kidneys, this study was explored on the CIR/I rats generated by performing intra-arterial suture occlusion of middle cerebral artery occlusion/reperfusion (MCAO/Reperfusion). The MCAO/Reperfusion rats were then investigated with histopathological changes and multiple *Aqps* expressions on the non-neural organs including kidneys, liver, colon and stomach. Furthermore, the rat kidneys were examined with protein expressions of Aqp1, Aqp2, Aqp3 and Aqp4 by using immunohistochemistry.

2. Materials and methods

2.1. Rat and MCAO/Reperfusion model

Male rats (Sprague Dawley, 230–250g) were supplied by Dashuo experimental animal Co. Ltd. (Chengdu, China) (certificate number: SCXK-2020-030). They were housed with standard laboratory chow and water for free at 22 ± 2 °C, 60 ± 5 % of the humidity, and a cycle of 12 h light/dark for the duration of the study. All protocols used in the research were approved by the Ethics Committee of Northwest University for Animal Experimentation (ACCNU-2020-0016). All procedures were performed based on the Guidelines for Animal Experimentation of Northwest University and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Following adaptive feeding for a week, the rats were assigned with sham (CON) and MCAO/Reperfusion group with eight rats in each for the study. In MCAO/Reperfusion group, the rats were injuried with focal cerebral ischemia by performing intraluminal filament occlusion at left middle artery. The detailed has been illustrated in our previous study [13]. Briefly, rats anesthetized (avertin, 125 mg/kg, ip) (T48402, Macklin Biochemical Technology Co., Ltd., China), were blocked on middle cerebral artery by using a nylon suture inserted from external to internal carotid artery. After ischemia for 60 min, reperfusion was launched by removing the intraluminal nylon suture. Rats were then housed in cages supplied with diets for free. After 24 h, neurological test was determined by using Longa's score. Thereafter, all rats were sacrificed and collected with blood and the tissues including brains, kidneys, livers, colons and stomachs. Brains were carefully removed rapidly and used for assessment of brain edema and TTC staining. Part of organ tissues were fixed by using 10 % formalin and part were kept in −80 °C used for RNA extraction.

2.2. Neurological function assessment

Longa's score was used based on the rat movement including paralysis of the contralateral forelimb, turning or tilting to the contralateral while walking. Neurological function score was assessed from 0 to 5 scale. 0: no deficits; 1: incomplete extension of forepaw; 2: shifting to the paralyzed side or even turning when walking; 3: collapsed to the paralyzed side when walking or no spontaneous motor activity; 4: unable to move autonomously; 5: loss of consciousness or dead. Two researchers scored the animals in a blinded manner. To generate MCAO/R rats with similar injury, the MCAO/R rats with the Longa's score at 4 or more were removed, and those at 2 to 3 were selected and used for the study. In the end, eight rats in each group were investigated for the study.

2.3. Brain edema assessment

The rat brain was dried at 110 °C for 24 h, and followed to be measured with dry weight. The brain edema was analyzed based on

the formulation:

$$\frac{(\text{wet brain weight} - \text{dry brain weight})}{\text{wet brain weight}} \times 100\%.$$

2.4. The infarct volume determination

Briefly, the rat brain was cut into 5 slices in coronal position. The rat brain slice was stained in 4 % of dye solution which was 2,3,5-triphenyltetrazolium hydrochloride (TTC) (G1017, Servicebio, Wuhan, China) in the dark at 37 °C for 30 min and photographed in a background for later analysis of the infarct size. The infarct volume in each brain was the sum of infarct volume for each slice.

2.5. Staining with hematoxylin and eosin

The rat kidneys were fixed and paraffin-embedded, and then the section was cut at 0.45 μm for subsequent staining. Hematoxylin & Eosin staining (H&E) was carried out on the rat kidneys, livers, colons and stomachs. The procedure was described in our previous study [12].

2.6. Immunohistochemistry staining

The immunohistochemistry staining was used to assess the expression of Aqp proteins on kidney tissues by using the method illustrated in our published study [12] Briefly, the dewaxed section was heated in citrate buffer with pH6 for 15 min, and blocked in 3 % of goat serum, followed to be incubated in primary antibody solution overnight at 4 °C. They were rabbit anti-aquaporin 1 (GB11310-1), anti-aquaporin 2 (GB112259), anti-aquaporin 3 (GB11395) and anti-aquaporin 4 (GB11529) with the dilution in 1:400. The secondary antibody, biotinylated IgG antibody (G1213) with the dilution at 1:200 was used and incubated with the section at 37 °C for 30 min. The kidney tissue sections were further developed by Strepavidin-Biotin-Complex with DAB (G1211), and the section was imaged. All the above products used were supplied by Servicebio Technology Co., Ltd. in Wuhan, China.

2.7. Biochemical analysis

The rat bloods collected were centrifuged at room temperature with 3500 rpm for 10 min. Then the supernatant was achieved and stored in freezer at −80 °C. The level of serum urea nitrogen, uric acid, and creatinine was detected by using an automatic biochemical analyzer (Rayto, China). The levels were analyzed by enzyme coupling colorimetric method.

2.8. RNA extraction and cDNA synthesis

TRIzol reagent (B511311) was purchased from Sangon Biotech Company, Ltd in Shanghai, China. A tissue grinder was manufactured by Ningbo Scientz Biotechnology Company Ltd. in Nignbo, China. The rat kidney samples in TRIzol were homogenized by the grinder at 4 °C. The RNA was isolated and cDNA was synthesized by using method described in our previous study [9]. The RNA was checked with quality according to the 260/280 nm ratio (1.8–2.0), and cDNA was synthesized following the protocol outlines in our previous research [9].

2.9. Real time quantitative PCR analysis

RT-qPCR experiments were carried out by using the cDNA synthesized with pair of primers used in previous research [9]. In each sample, the relative expression of the AQP subtype was normalized to the expression of an endogenous gene *Hprt*. RT-qPCR was carried out with 60 ng of cDNA for each in SYBR Green master mix (RK21203, abconal, Wuhan, China), 0.2 μM of pair of primers. The thermal cycling for RT-qPCR reactions was set with denaturation (95 °C for 3 min), 40 cycles of reactions (95 °C for 5s). Primer annealing was set at 60 °C for 30s. Non-specific amplification was checked regarding to the melting curve formed during RT-qPCR reaction. The gene expression was expressed relative to *Hprt* by using $2^{-\Delta\Delta Ct}$ method. The expression for each gene is the mean levels of 3 rats at least for each group.

2.10. Statistical analysis as well as principal component analysis (PCA)

Past3 software was used for the PCA analysis. The data is expressed as means ± SD. By using GraphPad Prism version 6.0 (GraphPad Software, Inc, San Diego, CA, USA) and SPSS 19.0 software, the statistical analysis was performed. ANOVA analysis was performed for the comparisons and the p values were subjected further to Bonferroni's correction. $p < 0.05$ (*) was thought to be statistically significant. All p values shown in the study are referred to the post-correction values.

3. Results

3.1. CIR/I shown in the MCAO/reperfusion rats

To date, MCAO/R*eperfusion* injury is the most widely accepted rodent model. The reperfusion after 1 h occlusion has been widely used in many studies [14]. In this study, the sham (CON) rats and MCAO/R*eperfusion* rats were assessed with neurological function, brain edema and cerebral infarction.

To assess the neurological function, Longa's score was used in terms of the rat behaviors. The CON rats showed normal behaviors with the Longa neurological function score at 0. Whereas the MCAO/Reperfusion rats had shifting/collapsed to the paralyzed side or even turning when walking. The score was around 2 or 3 (Fig. 1A), which was significantly different from that of the CON rats ($p = 0.04$, $^*p < 0.05$). Above results indicated that the MCAO/Reperfusion resulted in the neurological deficit to the rats.

In addition, brain edema, another important indicator, was evaluated as well. Fig. 1B showed that the percentage of brain water was 76.18 % in the CON rats and was 80.98 % in the MCAO/Reperfusion rats. Thus, there was 4.80 % higher in the MCAO/Reperfusion rats than those in the CON rats ($p = 0.05$, $^*p < 0.05$), indicating the formation of brain edema in the MCAO/Reperfusion rats.

Furthermore, as the primary endpoint in the animal model, the size of cerebral infarction is an essential indicator to evaluate the brain injury induced [15]. In general, method of TTC staining could give more quick detection for cerebral infarction in terms of a

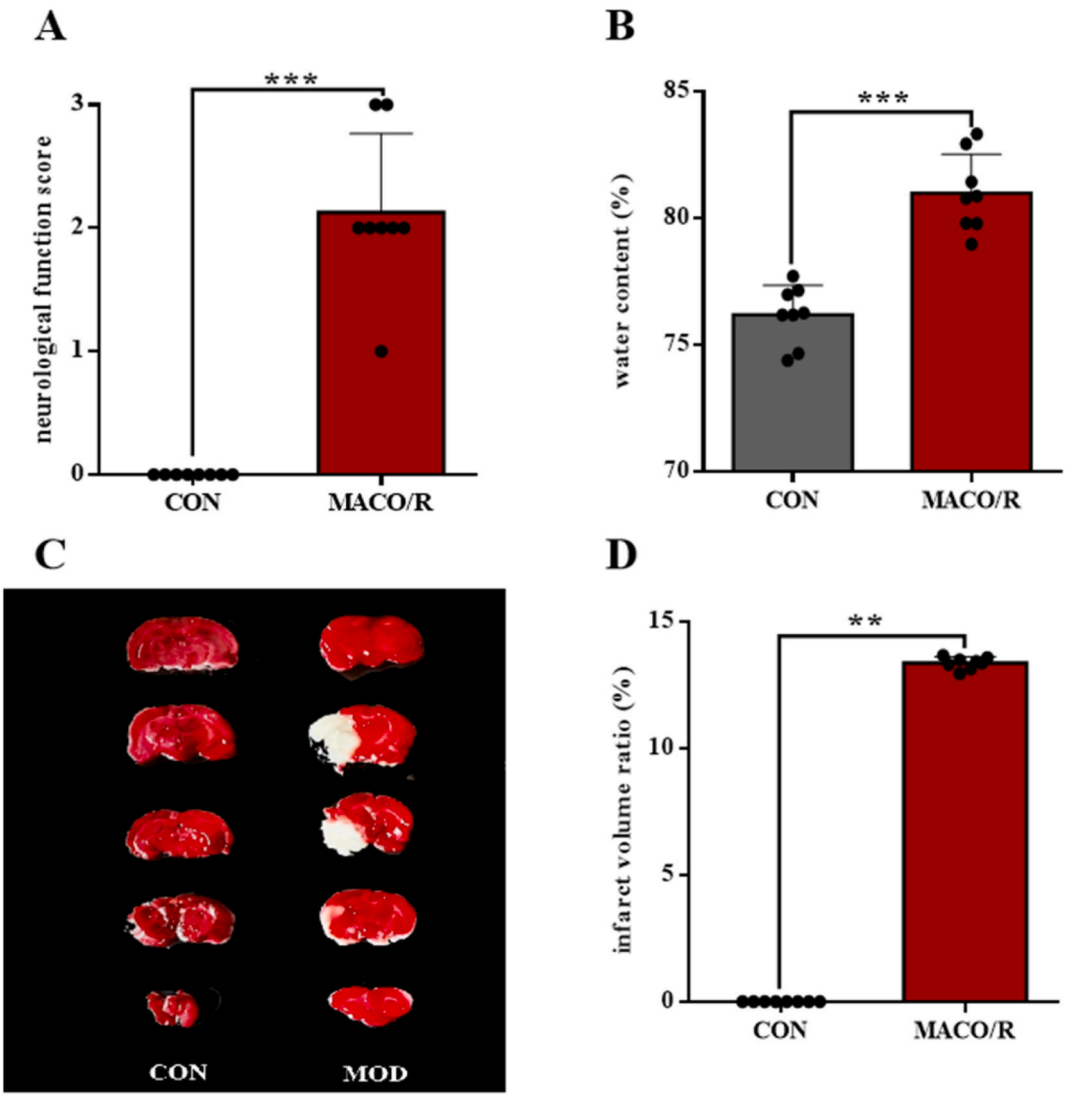

Fig. 1. Cerebral ischemia-reperfusion injury shown in the rats involved in MCAO/Reperfusion group. (A) Neurological scores in the rats; (B) Water content in the rat brains; (C) TTC staining on the rat brain sections; (D) Cerebral infarct volume ratio. CON represents the rats involved in the sham group; MCAO/Reperfusion represents the model rats generated by performing MCAO/Reperfusion. (n = 8). The value with $^*p < 0.05$ means statistically significant.

redox reaction. In stroke rats, the staining provided consistent results to H&E staining at 24 h after reperfusion [16]. In our study, the rats were assessed with brain infarct size at 24 h after the performance of MCAO/Reperfusion, which has been widely used in many studies [17,18].

TTC staining was performed by the brain being cut into five slices in coronal position and stained immediately. The brain tissues without any infarction would be red, and the infarct part would be white. As shown in Fig. 1C, there was no infarct in the CON rats indicated by all the sections colored with red. But there were some white sections shown on the brain tissues collected from the MCAO/Reperfusion rats. The marked lesions were observed on the left cerebral hemisphere. The infarct volume ratio in the MCAO/Reperfusion group was around 13 % and was significant different with that in the CON rats ($p = 0.01$, $^*p < 0.05$) (Fig. 1D). This demonstrated that the cerebral infarct was induced in the MCAO/Reperfusion rats.

Overall, above results strongly confirmed that the CIR/I injury was successfully generated in the MCAO/Reperfusion rats.

3.2. Histopathological changes and blood biochemical profiles for kidneys of the MCAO/reperfusion rats

To investigate the potential injury on the non-neural organs, H&E staining was first carried out on the paraffin-embedded rat tissues including kidneys, livers, colons and stomachs. No obvious pathological changes could be observed in the livers, colons and stomachs (available upon request). In the kidneys, the normal architecture was shown in the CON rats (Fig. 2A) and often in MCAO/R*eperfusion* rats (Fig. 2B). But occasionally, minor tubules dilation could be found in the kidneys collected from the MCAO/R*eperfusion* rats.

AKI is defined as a sudden decline of tubular function and glomerular filtration, leading to the accumulation of nitrogenous wastes including urea. Thus, the levels of serum urea nitrogen (UREA), serum creatinine (CREA), and serum urea acid (UA) were detected in the rats collected from the two groups (Fig. 2C). There were clear increases with the levels of CREA, UA and UREA in the MCAO/Reperfusion rats compared to the CON rats. Particularly, there was a statistical difference with the levels of CREA between the MCAO/Reperfusion rats and the CON rats ($p = 0.05$, $^*p < 0.05$).

Therefore, there might be a rapid injury that occurred in the MCAO/Reperfusion rat kidneys.

3.3. Altered Aqps expressions shown in kidneys, livers, colons and stomachs of the MCAO/reperfusion rats

To look at alteration of the *Aqps* expressions in the four organs, we used RT-qPCR to quantify mRNA levels of multiple Aqps subtypes including *Aqp1 ~ Aqp9* and *Aqp11*. *Hprt* was used as a reference gene by using the method applied in our previous study [9]. The transcription level for each gene was determined by the $2^{-\Delta\Delta CT}$ method and showed in Fig. 3A.

Compared to the CON rats, the MCAO/Reperfusion rats showed down-regulation in the kidneys on most of the *Aqp* subtypes, excluding *Aqp8* and *Aqp9* with less up-regulations. Among them, the down-regulation was more prominent on *Aqp1*, *Aqp2* and *Aqp3* with statistical significance. In the livers, the MCAO/Reperfusion rats had obvious up-regulation on *Aqp8*, but minor changes on the others compared to the CON rats. In the colons and stomachs, there were less difference on most of the *Aqp* subtypes between the CON rats and the MCAO/Reperfusion rats.

To look at the highest impact on the organs, PCA was applied on levels of the *Aqps* subtypes for discrimination. The PCA analysis are

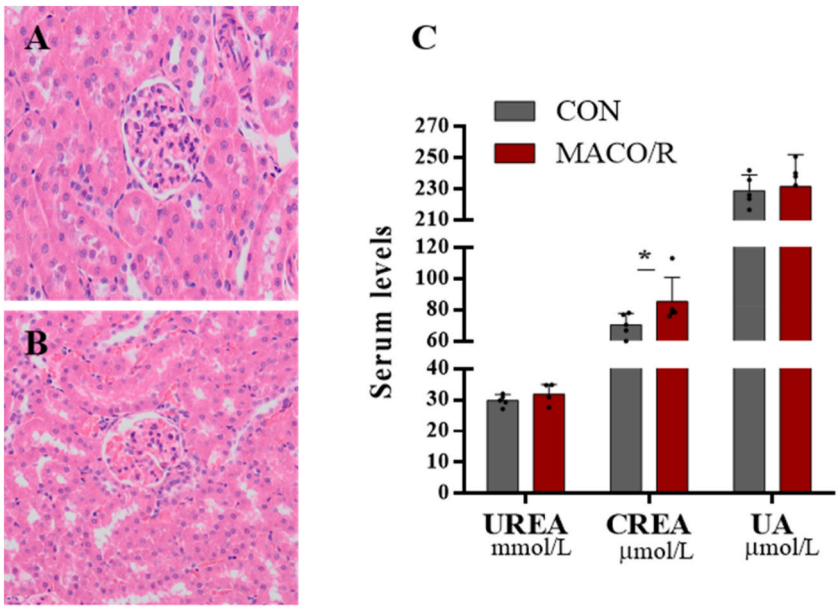

Fig. 2. Observation on the kidneys collected from sham (CON) and MCAO/Reperfusion rats. H&E staining for the CON rats (A) and MCAO/Reperfusion rats (B) (original magnification 200×); (C) Serum levels of UREA, CREA and UA in the rats. (n = 5).

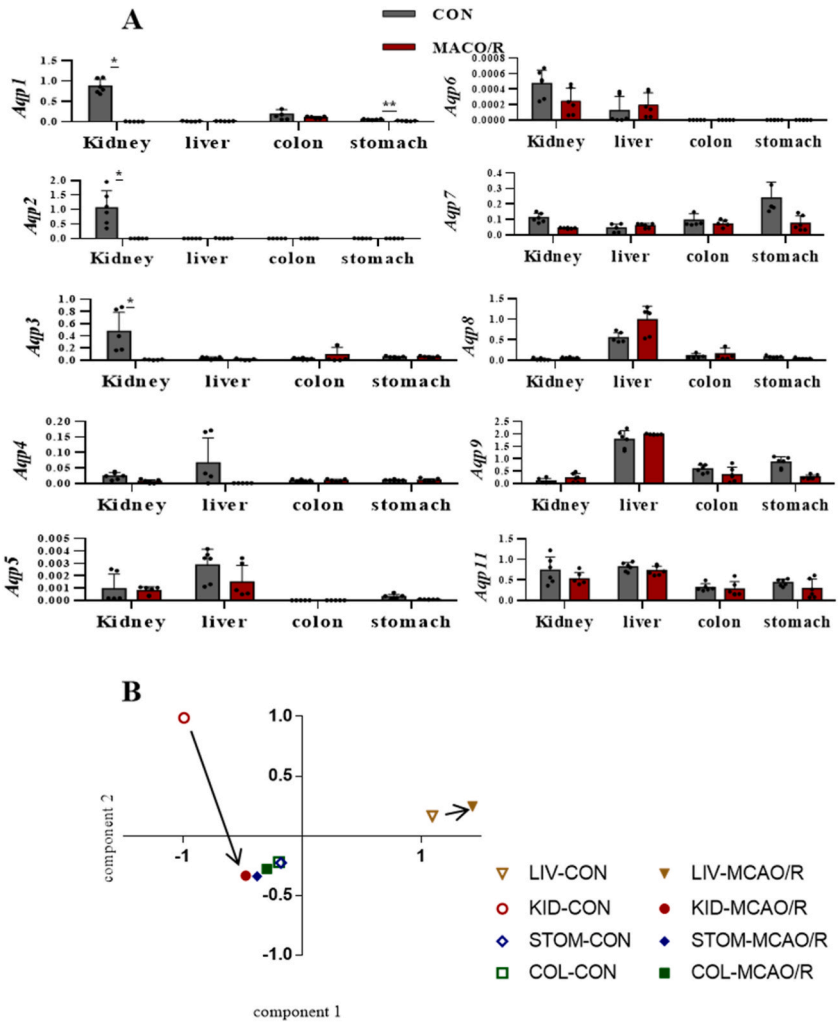

Fig. 3. Relative mRNA expression levels of multiple *Aqps* in the kidneys, lives, colons, and stomachs of the sham (CON) rats and the MCAO/Reperfusion rats. (A) Levels of *Aqps* transcripts are expressed as relative values to *Hprt* standard. Data shown as means ± SD (n = 6). (B) PCA results based on the multiple *Aqps* expressions. The value with $*p < 0.05$ means statistically significant.

described in Fig. 3B. It shows four evident clusters among the species including the kidneys, livers, colons and stomachs collected from the CON and MCAO/Reperfusion rats. The same cluster represents similar *Aqps* profile displayed in the organ. The farther distance implied the greater difference with the *Aqps* profile between the CON rats and the MCAO rats.

For the CON rats, there were clear differences among the livers, kidneys, and colons and stomachs, which are in three different clusters. For the MCAO/Reperfusion rats, the livers, colons and stomachs were in the same clusters as those for the CON rats, suggesting their similar status between the two groups. However, the kidneys collected from the MCAO/Reperfusion rats are localized in different clusters with those obtained from the CON rats. Comparably, the kidneys of the MCAO/Reperfusion rats are far away from those of the CON rats. Thus, the distinct difference with the *Aqps* alteration profile was displayed in kidneys between the CON rats and the MCAO/Reperfusion rats.

3.4. Decreased Aqps protein expressions in the kidneys of the MACO/R rats

As the significant decrease on mRNA expression was shown in the kidneys, Aqp1, Aqp2, Aqp3 and Aqp4 protein expression was investigated by immunohistochemistry. Protein expression was examined in cortex, outer and inner medulla areas of the kidneys.

In the CON rat kidneys, intensive Aqp1 staining was observed at the proximal straight/convoluted tubules in cortex area of the kidneys, and at the tubules in the renal outer medulla and inner medulla areas (Fig. 4A). In the MCAO/Reperfusion rat kidneys, the staining was slightly reduced at the corresponding parts (Fig. 4B).

In Fig. 4C, Aqp2 was labeled in the collecting duct of the CON rats, and abundantly expressed at the apical membranes of principal cells in the renal cortex, outer medulla and inner medulla areas of the kidneys. Whereas, in the MCAO/Reperfusion rats, the Aqp2

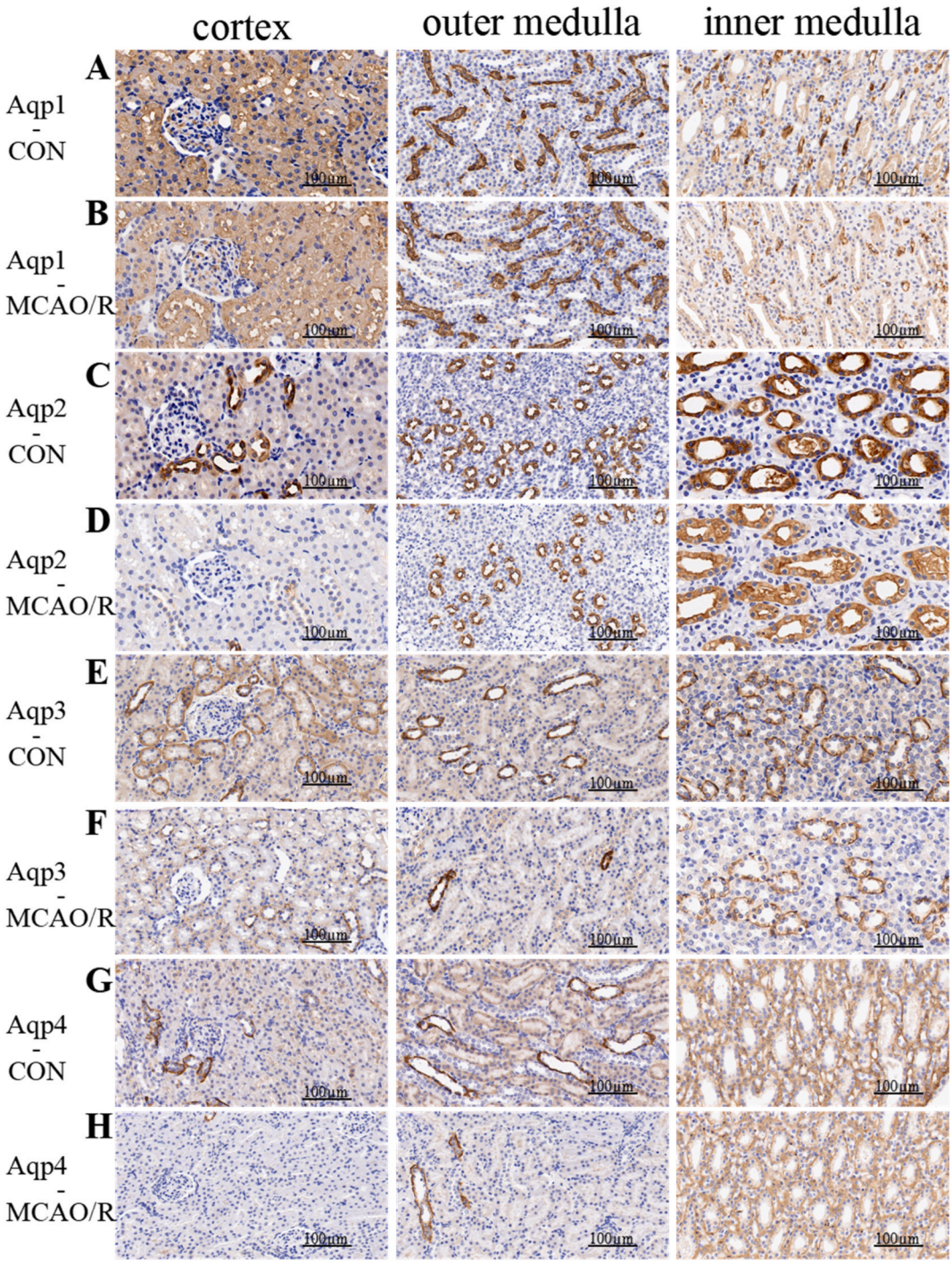

Fig. 4. Representative images of IHC staining for Aqps in sections of kidneys collected from the sham (CON) rats and the MCAO/Reperfusion rats. (A) Intensive Aqp1 staining shown in the CON rats; (B) Reduced Aqp1 staining shown in the MCAO/Reperfusion rats; (C) Abundant Aqp2 staining shown in the CON rats; (D) Less Aqp2 staining shown in the MCAO/Reperfusion rats; (E) Extensive Aqp3 expression shown in the CON rats; (F) Weak Aqp3 expression shown in the MCAO/Reperfusion rats; (G) Abundant Aqp4 labeling shown in the CON rats; (H) Light Aqp4 labeling shown in the MCAO/Reperfusion rats. (original magnification 200×).

abundance was markedly less in the renal cortex area (Fig. 4D). In the outer medulla and inner medulla area, the staining was somewhat weakened.

For the Aqp3, there was constitutive expression in the CON rats (Fig. 4E) at the basolateral membrane of principle cells on the

convoluted tubules and collecting duct in the cortex area, and collecting duct in the outer medulla and inner medulla areas. In the MCAO/Reperfusion rat kidneys (Fig. 4F), the Aqp3 staining appeared to be lower at the corresponding parts in the renal cortex, outer medulla and inner medulla areas.

Similar as for the Aqp3, the Aqp4 protein expression in the CON rats (Figure G) was labeled at the basolateral membrane of principle cells on the convoluted tubules and collecting duct in the cortex, outer medulla and inner medulla areas. The MCAO/Reperfusion rats (Fig. 4H) had markedly less and weak Aqp4 labeling on the convoluted tubules and collecting duct in the cortex area and outer medulla area, but there was a similar staining in the inner medulla area between the CON rats and the MCAO/Reperfusion rats.

4. Discussion

To date, CIR/I has been shown to cause damage through complex mechanisms including excessive production of reactive oxygen species (ROS), and pro-inflammatory mediators (IL-6, IL-1β, TNF-α, chemokines), etc [19]. These could not only damage the brain directly but also cross the damaged blood-brain barrier and enter the systemic circulation to make possible secondary injury on non-neuronal organs [18]. During the cardiac cycle, brain and kidney are exposed in a large amount of fluid flow. They act together to control fluid homeostasis between cells via modulating the balance of water and sodium [6]. As the primary organ to control water balance and to remove toxins by filtering blood, kidney is in the frontline for induction of damage as a result of the release of ROS and pro-inflammatory mediators following CIR/I. Thus, it is not surprise that AKI has been reported as one of the most common complications after stroke [20]. Consistently, our results showed that the kidney was in the highest risk to be injured following the CIR/I in terms of the *Aqps* alterations. Furthermore, down regulation of Aqp1, Aqp2, Aqp3 and Aqp4 was observed in the kidneys of the MACO/R rats.

Aqp1 protein is abundant on proximal tubule at the apical and basolateral plasma membrane, and descending vasa recta and thin limbs of Henle. Its main function is constitutive absorption of more than 70 % of water in the glomerular filtrate [21]. AQP1 mutation with loss-of-function resulted in an impaired function to concentrate urine when human was challenged by water deprivation. Mice defective with Aqp1 exhibited polyuria resulting in the formation of hypertonicity in the kidneys [22]. Furthermore, the Aqp1-KO mice had lower glomerular filtration rate and renal blood flow, accompanied by higher urinary sodium excretion and fractional sodium excretion compared to the wild type mice during endotoxemia [23].

Aqp2 protein plays a key role to maintain water balance in human body through regulating urine concentration. It is widely expressed in the principal cells of collecting duct from the cortex to the inner medulla. Aqp2 deletion in the mouse connecting tubules resulted in a increased urine volume with 1.5 fold but decreased urine osmolality with similar fold [24]. The impairment of Aqp2 is thought to be a main mechanism in charge of nephrogenic diabetes insipidus [25]. Notably, Aqp2 expression has been known to be regulated by arginine vasopressin secreted from the posterior pituitary gland [26], indicating the direct interaction between brain and kidney.

Aqp3 and Aqp4 are both distributed in the basolateral membrane of principle cells located in collecting duct of the cortex as well as outer medulla [22]. They represent a potential exit pathway from cell to the interstitium for water entering the cytoplasm via Aqp2. Specific deletion of Aqp3 in collecting duct obviously destroyed kidney function and significantly aggravated kidney damage indicated by activating renal oxidative stress, apoptosis and inflammation following ischemia reperfusion [27]. Aqp4-null mice appeared normal with respect to the kidney function, but the Aqp3/Aqp4 knockout mice displayed very weak urinary function than the mice with Aqp3-knockout [8]. Thus, the reduction of Aqp1, Aqp2, Aqp3 and Aqp4 could decrease urinary concentration ability, and result in a severe reduction in glomerular filtration rate and a variable fall in renal blood flow [20].

Moreover, the Aqps not only mediates water reabsorption but also play multiple functions by permeating monovalent cations, hydrogen peroxide, carbon dioxide, nitric oxide and ammonia [28]. For example, AQP1 is known to influence cell migration, angiogenesis, and proliferation So, in LPS-induced HK-2 cell, over-expression of the Aqp1 gene was reported to reduce the release of inflammation factors and alleviated cell apoptosis [29]. Aqp3 also facilitates movement of glycerol and H_2O_2 through the cell membrane, which modulates signaling pathway and influences cell functions such as cell proliferation, migration and apoptosis [30]. In the rodent models of renal ischemia/reperfusion injury, down-regulation of Aqp1, Aqp2, Aqp3 and Aqp4 has been found to be main characteristics and correlated with the reduction of the kidney functions [31–35]. Thus, AQPs have been implicated in multiple disorders such as epilepsy, brain edema, metabolic disorders, and inflammation. Researches believed that blocking AQPs with drugs could help treat these conditions by occluding the intrasubunit water-channel pore at the intracellular or extracellular vestibule. The small-molecule AQP inhibitors have been extensively studied for many years but so far no pore-blocking drugs for any AQP have been approved for clinical use [36].

In fact, dysregulation of AQPs has received extensive attention in the study of CIR/I, particularly in the development of brain edema, which was observed in the MCAO/Reperfusion rats in our study. With the growing efforts to elucidate mechanisms underlying the formation of brain edema, AQP4 was identified to be a valid therapeutic target.

In central nervous system (CNS), astrocyte is the most well-known glial cell type, which plays a key role in multiple functions, such as to maintain neuronal function, to control ion concentration, to regulate the blood-brain barrier, to support neuronal nutrition and metabolism, and to participate immune response [37]. AQP4 is the principle of AQPs family in CNS, being highly expressed abundantly in astrocyte membranes with lateral diffusion or at end-feet [38]. When AQP4 is diffusely expressed throughout the astrocyte membrane, it can cause cytotoxic edema by modulating cell membrane permeability for water molecules. However, when it is localized to the end-feet of astrocytes adjacent to cerebral microvasculature, it contributes to clearance of vasogenic edema. Recent studies have shown that ischemic stroke can lead to the rapid occurrence of cytotoxic edema, followed by the formation of vasogenic

edema [39]. During this process, astrodegeneration could arise from astroglial morphological atrophy, cell death, and/or loss of function. The AQP4 expression was observed to have been relocated from the perivascular end-feet to the entire astrocytic membrane. However, the subcellular relocalization was not always result in a change in AQP4 protein levels [38]. This suggested that the translocation of AQP4 had a greater impact on brain edema and astrocyte function than its expression level in the brain. Indeed, TGN-020, an AQP4 inhibitor, did not affect extracellular space volume dynamics induced in the Aqp4+/+ rats. In contrast, pharmacological inhibition of AQP4 translocation could effectively alleviate brain edema of post-stroke mice during the early acute phase and promote functional recovery of astrocytes in injured rats [40]. The change in perivascular AQP4 localization at the end-feet of astrocytes is also associated with various neurodegenerative diseases, including ageing, cerebrovascular disease, CNS injury, and Alzheimer's disease [41]. The subcellular relocalization was discovered to be in response to environmental changes, such as the influx of calcium, and hypoxia condition in primary cortical astrocytes [38,42].

Similarly, other AQP subtypes have been found to be regulated through subcellular relocalization. For instance, in response to the anti-diuretic hormone and arginine vasopressin, AQP2 relocated to the apical membrane in the kidney collecting duct cells. On the other hand, both AQP3 and AQP4 are localized to the basolateral membrane of kidney collecting duct principal cells, but they appeared to be transported to plasma membrane via vesicle pools of mutually-exclusive post-Golgi. In HEK293 cells, AQP4 translocation was found to be associated with tonicity-dependent changes in the environment that led to calcium-dependent, calmodulin-dependent, and PKA-specificity [43]. Additionally, it has been discovered that AQP4 is also present in vesicles within the cytoplasm. When exposed to hypotonic conditions, the AQP4-eGFP quickly translocated from intracellular vesicles to the HEK293 cell surface [44]. In our study, Aqp1 ~ Aqp4 were found in the cortex, inner and outer medulla of the rat kidneys. Interestingly, in the MCAO/Reperfusion rat kidneys, the loss of expression occurred mainly at the cortex but not at other locations. The evidence implies that a ubiquitous regulatory mechanism is involved in AQP subcellular relocalization across the mammalian AQP family [45]. Therefore, an alternative route to target AQP function would be explored through the translocation mechanism of AQPs, instead of the traditional pore-blocking

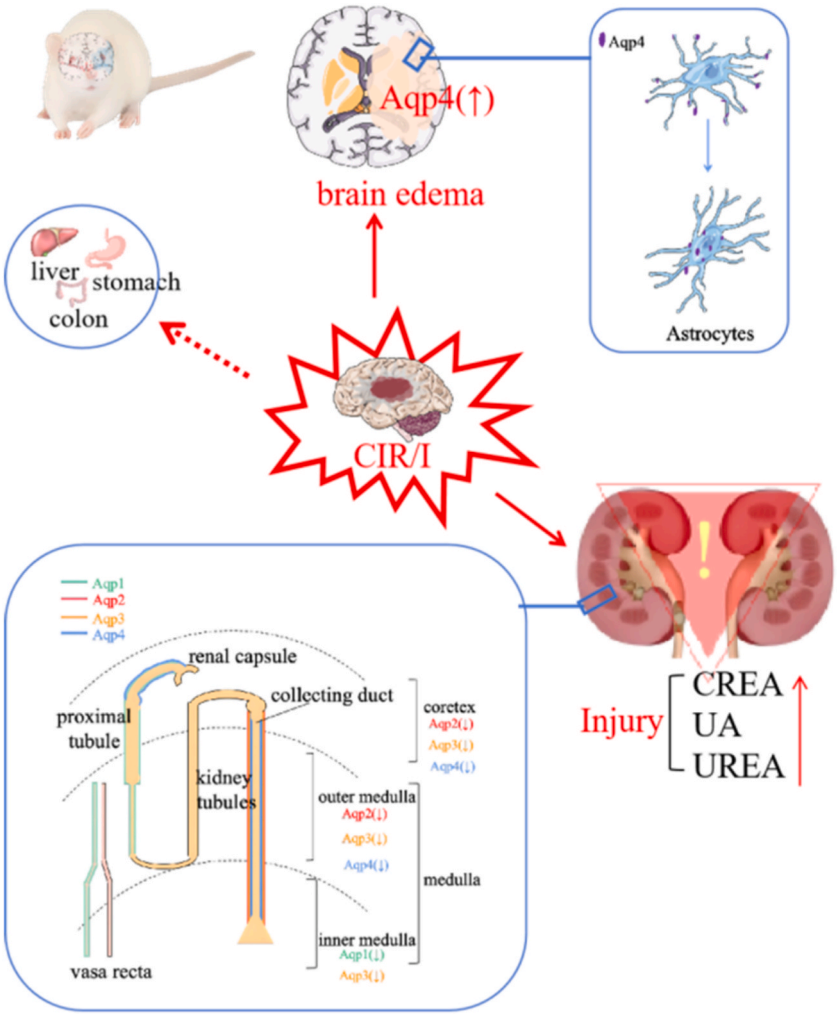

Fig. 5. Schematic kidney damages resulted from cerebral ischemia/reperfusion.

approach. In the study, there was a regulatory sub-cellular relocalization of renal Aqp2, Aqp3, and Aqp4 in MCAO/Reperfusion rats, which might offer possibilities to develop AQP-targeted therapeutics for AKI.

Moreover, the AKI after stroke often occurs rapidly within the first week [4]. Currently, the routine diagnosis relies on increase of serum creatinine levels based on Kidney Disease Improving Global Outcomes clinical practice guidelines [46]. Consistently, our study showed that the MCAO/Reperfusion rats had increases on the serum CREA, UA and UREA levels. However, it is still more challenging to predict the abrupt insult on the kidney due to limited sensitivity and specificity [47]. Thus, researchers are committed to finding new markers for diagnosis to prevent the occurrence and development of AKI. In this study, down-regulation of the Aqp2, Aqp3 and Aqp4 was shown on mRNA as well as protein levels. We presume that the alterations represented the disturbance of the fluid homeostasis arisen in the MCAO/Reperfusion rat kidneys. The Aqps down-regulation could result in the reduction of glomerular filtration rate and lead to structural alteration in renal tubular epithelia (Fig. 5).

5. Conclusion

By looking at the *Aqps* alteration profile, the kidneys were found to be at the highest risk to be injured following the CIR/I. Down-regulation of Aqp2, Aqp3, and Aqp4 was involved in the acute kidney injury induced by the CIR/I. We presume that Aqp2, Aqp3 and Aqp4 could be used as early diagnostic markers and to facilitate the development of effective drugs for kidney protection in the stroke treatment.

Ethical approval

All procedures used in the study were approved by the Ethics Committee of Northwest University for Animal Experimentation with the reference number ACCNU-2020-0016.

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Data availability statement

All data used in this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Meng Dai: Methodology, Investigation, Data curation. **Jinglei Yang:** Investigation. **Zhaoyang Wang:** Investigation, Data curation. **Fangli Xue:** Investigation. **Yourui Wang:** Investigation. **Enjie Hu:** Data curation. **Yunyun Gong:** Writing – review & editing. **Michael N. Routledge:** Writing – review & editing. **Boling Qiao:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

AKI acute kidney injury
Aqps Aquaporins
CIR/I cerebral ischemia-reperfusion injury
H&E staining Hematoxylin and eosin staining
Hprt hypoxanthine phosphoribosyltransferase
IHC Immunohistochemistry; LPS, lipopolysaccharide
MCAO/Reperfusion middle cerebral artery occlusion/reperfusion
NC Normal control
PCA principal component analysis
RT-qPCR Reverse transcription quantitative polymerase chain reaction
SD rat Sprague Dawley rat
TTC 2,3,5-triphenyltetrazolium hydrochloride.

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