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ORIGINAL PAPER

The Involvement of Mitochondrial Biogenesis in Selenium Reduced Hyperglycemia-Aggravated Cerebral Ischemia Injury

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Abstract

Selenium has been shown to possess antioxidant and neuroprotective effects by modulating mitochondrial function and activating mitochondrial biogenesis. Our previous study has also suggested that selenium protected neurons against glutamate toxicity and hyperglycemia-induced damage by regulating mitochondrial fission and fusion. However, it is still not known whether the mitochondrial biogenesis is involved in selenium alleviating hyperglycemia-aggravated cerebral ischemia reperfusion (I/R) injury. The object of this study is to define whether selenium protects neurons against hyperglycemia-aggravated cerebral I/R injury by promoting mitochondrial biogenesis. In vitro oxygen deprivation plus high glucose model decreased cell viability, enhanced reactive oxygen species production, and meanwhile stimulated mitochondrial biogenesis signaling. Pretreated with selenium significantly decreased cell death and further activated the mitochondrial biogenesis signaling. In vivo 30 min of middle cerebral artery occlusion in the rats under hyperglycemic condition enhanced neurological deficits, enlarged infarct volume, exacerbated neuronal damage and oxidative stress compared with normoglycemic ischemic rats after 24 h reperfusion. Consistent to the in vitro results, selenium treatment alleviated ischemic damage in hyperglycemic ischemic animals. Furthermore, selenium reduced the structural changes of mitochondria caused by hyperglycemic ischemia and further promoted the mitochondrial biogenesis signaling. Selenium activates mitochondrial biogenesis signaling, protects mitochondrial structure integrity and ameliorates cerebral I/R injury in hyperglycemic rats.

Keywords Selenium · Hyperglycemia · Cerebral ischemia and reperfusion · Mitochondrial biogenesis · Oxidative stress

Abbreviations

I/R	Ischemia reperfusion
MCAO	Middle cerebral artery occlusion
SD rats	Sprague Dawley rats
ROS	Reactive oxygen species
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
NRF1	Nuclear respiratory factor 1
TFAM	Mitochondrial transcription factor a
UCP2	Uncoupling protein 2
SOD2	Mitochondrial superoxide dismutase 2
ANOVA	Analysis of variance
SD	Standard deviation
DMEM	Dulbecco's Modifid Eagle Medium
FBS	Fetal bovine serum
GPx	Glutathione peroxidase
CCK-8	Cell counting kit-8
HRP	Horseradish peroxidase
PVDF	Polyvinylidene fluoride membrane
PBS	Phosphate buffered saline
RFI	Relative fluorescence intensity
TEM	Transmission electron microscopy
TTC	2,3,5-Triphenyltetrazolium chloride
TUNEL	Terminal transferase biotinylated-dUTP nick end labeling
BCA	Bicinchoninic acid

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STZ Streptozotocin
DHE Dihydroethidine

Introduction

Cerebral ischemic injury leads to a high incidence of disability and mortality [1, 2]. Stroke patients are usually associated with metabolic diseases such as obesity, hyperglycemia and hyperlipidemia. Hyperglycemia is one of the most important risk factors closely related to the occurrence and progression of stroke [3]. Diabetic patients are much more likely to experience cerebral stroke [3–7]. It is of great significance to search for preventive and therapeutic approaches that could effectively slow down or reduce diabetes-aggravated ischemic injury.

Selenium is a trace element essential to maintain the function of cells in most animals including humans [8]. Selenium supplementation increases the expression of selenium-dependent enzymes [9]. Selenium treatment increases the activity of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) to decrease oxidative stress [9, 10]. Published papers showed that selenium treatment could reduce mitochondrial oxidative stress in Parkinson's disease and cerebral ischemia in rat hippocampus [11, 12]. Our previous studies have suggested that selenium reduced cerebral ischemic infarct volume through prereserving mitochondrial function, stimulating mitochondrial biogenesis, regulating mitochondrial dynamic balance between fission and fusion, and blocking mitochondria-mediated cell death pathways [10, 13, 14].

The objective of this study is to define whether the protective effect of sodium selenite against hyperglycemia-aggravated cerebral ischemic injury is associated with activating the mitochondrial biogenesis pathway and preserving mitochondrial structural integrity. The results demonstrated that selenium protected neurons against hyperglycemia-aggravated cerebral ischemic damage. The protective effect was associated with activation of the mitochondrial biogenesis signaling pathway and preserving mitochondrial structure.

Materials and Methods

Cell Culture and Reagents

Murine hippocampal neuronal HT22 cells were cultured with Dulbecco's Modified Eagle Medium (DMEM)/F12 (HyClone laboratories, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, HyClone Cell Culture and Bioprocessing) and 1% penicillin/streptomycin (HyClone laboratories). HT22 cells were cultured at 90% relative humidity in 5% CO_2 at 37 °C. The culture medium was renewed every 24 h. Sodium selenite (Sigma, cat. 214485, 100 nM) was added to the culture medium 24 h prior to the induction of oxygen deprivation (OD), which is a model for hypoxia, or OD plus addition of D-glucose (Sigma-Aldrich, St Lois, MO, 50 mM) (OD + HG), which is a model to mimic ischemia in hyperglycemic/diabetic condition. Sodium selenite was removed from the culture medium at the onset of hypoxia or high glucose intervention. OD was produced by filling the anoxic chamber (Thermo scientific series II, America) with N_2 and the final oxygen content in the incubator was maintained at 2.5 ± 1.0 nmol/ml. After 1 h of OD, the cells were reoxygenated and harvested at 24 h after the reoxygenation for viability and biochemical assays. The concentration of glucose during normal culture and reoxygenation was 17.5 mM.

Cell Viability Assessment

Cell viability was determined using Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Tech. Inc., Rockville, MD) according to the manufacturer's instruction. In brief, HT22 cells were cultured in 96-well plates (5000/well, Corning Inc., New York, NY). Cell viability was assessed at 24 h after the HT22 cells being exposed to OD and/or high glucose for 1 h using SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA).

TUNEL Staining

Terminal transferase biotinylated-dUTP nick endlabeling (TUNEL) assay (Kaiki, China) were performed in situ to detect neuronal cell death, according to the manufacturer instruction. Images were captured from five microscopic fields per group by a fluorescent microscope (400 ×, HPF) and presented as ratio of TUNEL-positivelu stained neuronal cells over number of total cells per high-power field.

Determination of ROS

After being exposed to 1 h hypoxia with 50 mM glucose and reoxygenated for 24 h, the cells were incubated with dihydroethidine (DHE, 50 umol/l) in the culture medium at 37 °C for 30 min. The cells were then washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde. Images were acquired under 400 × magnification using a fluorescent microscope. The results were presented as relative fluorescence intensity (RFI).

Western Blot Analysis

At the end of each experiments, the cells were harvested and rapidly homogenized with lysis buffer on ice. In animal study, the brain tissues were dissected from ischemic

cortices (penumbra) and homogenized after sham-operation, 30 min MCAO plus 24 h and 72 h reperfusion. The protein concentrations in each sample were determined using the BCA Protein Assay Kit (Beyotime, China). Equal amount of protein (50 μg/μl) was loaded onto 10% sodium dodecyl sulfate–polyacrylamide gels for electrophoresis, and then transferred onto a polyvinylidene fluoride membrane (PVDF, 0.45 μm, Millipore, USA). The membranes were immersed in 5% non-fat milk to block the non-specific binding sites and then incubated with primary antibodies at 4 °C overnight. The primary antibodies include anti-UCP2 (1:1000, Bioss, Beijing), SOD2 (1:1000, Bioss, Beijing), PGC-1α (1:1000, Abcam, Cambridge, MA, USA), NRF1 (1:5000, Abcam, Cambridge, MA, USA), TFAM (1:5000, CST, MA, USA), and β-actin (1:2000, Bioworld, USA). β-actin served as an internal control. On next day, the membranes were washed and incubated with secondary antibodies (goat anti-rabbit IgG, 1:3000 or goat anti-mouse IgG, 1:5000) for 2 h at room temperature. The blots were then developed by enhanced chemical luminescence (Thermo Fisher, USA). The relative densities of the target bands were quantified using the Photoshop and normalized to β-actin.

Animals and Reagents

Ninety-two male Sprague Dawley (SD) rats, weighing 200–220 g, were provided by the Experimental Animal Center of Ningxia Medical University (Yinchuan, China). Among them, 20 were used for TTC, 26 for histological staining, 33 for Western blot, and 13 for transmission electron microscopy (TEM). Detailed assignment of animals is given in Table 1. All experiments were conducted at Ningxia Medical University in strict accordance with the Chinese Laboratory Animal Use Guidelines and approved by institutional Animal Use and Care Committee at Ningxia Medical University. Animals were kept on laboratory chow and housed in standard conditions in a temperature controlled room (20–22 °C) with a cycle of 12 h light/dark. The animals were fasted at least 15 h and i.p. injected with streptozotocin (STZ, Sigma, USA, 60 mg/kg) to induce diabetic model. The animals blood glucose levels were checked at 3 days after STZ injection and those who has a level > 16.8 mmol/l were recruited to hyperglycemic group. Animals in sodium selenite-treatment group were injected daily i.p. with sodium selenite dissolved in PBS (0.4 mg/kg/day) for 4 weeks prior to the induction of cerebral ischemia. The animals were divided into 3 groups (Table 1) according to the blood glucose level and selecium treatment, i.e. (1) normoglycemic ischemia (NI); (2) hyperglycemic ischemia (HI); (3) hyperglycemic ischemia + sodium selenite treatment (HI + Se). Each group was further divided into sham, reperfusion at 24 h and 72 h subgroups.

Table 1 Summary of groups and sub-groups

Groups	Processing	TTC	Histo	WB	TEM
NI					
Sham	Saline + sham	3	5	3	3
R24 h	Saline + MCAO	4	6	4	3
R72 h	Saline + MCAO	0	0	4	0
HI					
Sham	STZ + saline + sham	0	0	3	0
R24 h	STZ + saline + MCAO	6	8	4	3
R72 h	STZ + saline + MCAO	0	0	4	0
HI + Se					
Sham	STZ + Se + sham	0	0	3	0
R24 h	STZ + Se + MCAO	7	7	4	4
R72 h	STZ + Se + MCAO	0	0	4	0

NI normoglycemic ischemia, *HI* hyperglycemic ischemia, *Se* sodium selenite, *R24 h and R72 h* reperfusion at 24 h and 72 h, *MCAO* middle cerebral artery occlusion, *STZ* streptozotocin, *TTC* 2,3,5-triphenyltetrazolium chloride, *Histo* histology, *WB* Western blot, *TEM* transmission electron microscopy

MCAO Model

Focal cerebral ischemia was induced by unilateral middle cerebral artiery occlusion (MCAO) according to previous methods [15, 16]. In brief, the common carotid artery, internal carotid artery and external carotid artery on the right side were exposed. A monofilament nylon suture (external diameter 0.28–0.38 mm) with a rounded tip coated with silicon was inserted into the right internal carotid artery from the common carotid artery until a faint resistance was felt to block the origin of the middle cerebral artery. After 30 min of MCAO, the intraluminal filament was withdrawn to restore blood flow. In the sham group, the same surgical procedure was performed without closure of the middle cerebral artery. We selected 30 min MCAO because previous studies has shown that the aggravating effects of hyperglycemia on ischemic brain damage could be clearly differentiated with this short length of ischemic insult [16].

Infarct Volume Assessment

The animal brains were removed and sliced into 2 mm thick coronary sections in an adult rat brain matrix (RWD, China). The sections were stained with 1.5% solution of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, USA) at 37 °C for 30 min, and then immediately fixed in 4% formaldehyde overnight. The images of TTC stained brain sections were captured with a digital camera into a computer. Normal tissue was stained as red and infarct area as white. The infarct area was measured by Image J program and the infarct volume was calculated using the following equation:

Percentage of hemispheric infarction volume = (contralateral area − ipsilateral non-infarct area)/contralateral area × 100%.

Evaluation of Neurological Deficits

Neurological deficit scores were assessed by an examiner who was blind to the experimental groups. The rats were subjected to a neurological examination after 0-, 24-, and 72-h reperfusion using a modified scoring system [17]. Grade 0, normal neurologic behavior; (1) unable to extend left forepaw completely; (2) crawling towards left; (3) falling to left; (4) failure or difficult to walk spontaneously and exhibiting depressed levels of consciousness. Rats who had scores 2 or above were considered as an indicator of successful induction of the MCAO. Six rats were excluded from the study because their neurological score equal or less than 1.

Tissue Preparation and Histological Evaluation

After neurological functional scores being assessed, the rats were euthanized and decapitated at 24 h after reperfusion. Their brains were immersed by 4% paraformaldehyde for 24 h. The brains were then dissected into 5 blocks, dehydrated and embedded in paraffin. The brain blocks were sectioned at 4-μm thickness using a microtome (LEICA RM2235, Wetzlar, Germany). The brain sections were either stained with Eosin–hematoxylin (H&E) or Nissl staining according to standard protocols. The results were examined under a light microscope and images were captured into a computer. Five microscopic fields (400 ×, HPF) in the cortical area of each animal were captured and used for counting and presented as average of pyknotic cells (H&E stained sections) or viable neurons (Nissl stained sections) per HPF.

Specimen Preparation for TEM

The penumbral cortical tissues (size: approximately 1 × 1 × 2 mm^3) were isolated and immediately fixed in 2.5% glutaraldehyde solution overnight at 4 °C, and post-fixed in 1% osmium tetroxideat room temperature for 2 h. The samples were then subjected to a graded ethanol dehydration, and infiltrated with epoxy resin. Ultrathin sections (60 nm) were cut and stained with uranyl acetate for 20 min and lead citrate for 5 min and then viewed using a transmission electron microscope (H7800, Hitachi).

Statistical Analysis

All data were represented as mean ± standard diviation (SD). Statistical analysis was performed using two-way analysis of variance (ANOVA) with SPSS 19.00. Tukey's post hoc test was used for multiple comparisons. A value of $p < 0.05$ was considered as statistically significant.

Results

Selenium Reduces Cells Death Under OD and OD + HG Conditions

To investigate the effect of selenium on HT22 cells under oxygen deprivation (OD) and OD plus high glucose (OD + HG) conditions, we performed cell viability and TUNEL assays. The results showed that HG per se reduced cell viability to 80% (Fig. 1a, c, $p < 0.01$ *vs.* Control) and increased TUNEL positively stained neurons to 186% of the control value (Fig. 1b, d, $p < 0.01$ vs. Control). OD (1 h) resulted in a 20% reduction in viability ($p < 0.01$ vs. Control) and a 147% increase of TUNEL positively stained neurons ($p < 0.01$ vs. Control). Combination of 1 h OD with 50 mM glucose caused a further reduction (44% of control value) in cell viability ($p < 0.01$ vs. OD) and an increase of TUNEL positively stained cells ($p < 0.01$ vs. OD). Treatment with selenium alone slightly increased cell viability ($p < 0.05$ vs. Control) and decreased TUNEL positively stained neurons ($p < 0.05$ vs. Control). Selenium pretreatment prevented high glucose-induced cell death as indicated by increased cell viability to 92.8% of the control values ($p < 0.01$ vs. HG) and significantly decreased ratio of TUNEL positive staining ($p < 0.01$ vs. HG). Selenium pretreatment prevented OD + HG induced cell death as reflected by increased cell viability to 94.8% of the control values ($p < 0.01$ vs. OD + HG) and significantly decreased ratio of TUNEL positive staining compared to OD + HG group ($p < 0.01$ vs. OD + HG).

Selenium Reduces ROS ROS Production Under OD and OD + HG Conditions

Levels of ROS were measured by DHE probe to assess the oxidative stress in HT22 cells after 1 h OD or OD + HG followed by 24 h reoxygenation (Fig. 2a). Relative fluorescence intensity (RFI) was summarized in the bargraph (Fig. 2b). The results revealed that there was almost no detectable ROS in unchallenged normal HT22 cells. HG exposure increased the production of ROS ($p < 0.01$ vs. Control). The level of ROS increased in the OD group as well ($p < 0.01$ vs. Control) and further increased in OD + HG group compared to the control and the OD alone groups ($p < 0.01$). Sodium selenite-treatment resulted in a decrease of ROS production ($p < 0.05$ vs. control). Treatment with sodium selenite to both HG and OD + HG groups significantly reduced the ROS levels ($p < 0.01$ vs. HG and $p < 0.01$ vs. OD + HG). To clarify the mechanism involved in selenium reducing the ROS production, we detected the protein levels of mitochohdnrial uncoupling

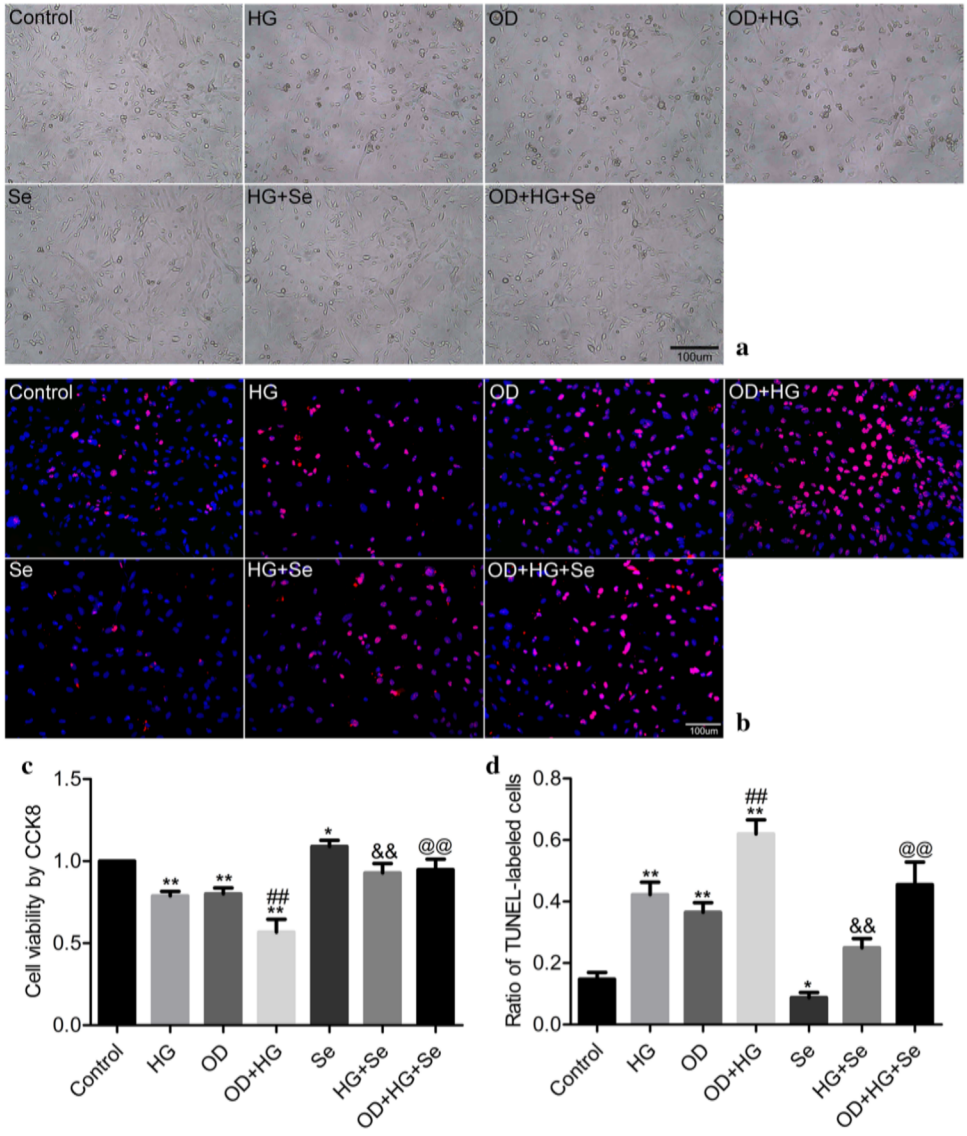

Fig. 1 Effects of selenium on cell viabilities and TUNEL staining in HT22 cells. **a** Representative photomicrograms of cell morphology, scale bar = 100 μm. **b** TUNEL staining. Red color, TUNEL staining; Blue color, DAPI nuclear staining. Scale bar = 100 μm. **c** Cell viability at 24 h of reoxygenation following 1 h of OD or 1 h OD plus 50 mM glucose (OD + HG). Se, sodium selenite 100 nM. **d** Quantification of TUNEL positively stained cells over total cell as marded by DAPI. Experiments were repeated 4 times with triplicates for each condition each time. Data are presented as mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* control group; $^{\#\#}p < 0.01$ vs. OD group; $^{\&\&}p < 0.01$ vs. HG group; $^{@@}p < 0.01$ vs. OD + HG group

protein 2 (UCP2) and mitochondrial superoxide dismutate (SOD2) by Western blot (Fig. 2c). As shown, after HG exposure, both UCP2 and SOD2 protein contents markedly decreased (Fig. 2d, e, $p < 0.01$ vs. Control). OD suppressed the levels of these antioxidant enzymes compared to control ($p < 0.01$ vs. Control). OD + HG further suppressed the levels of anti-oxidant UCP2 ($p < 0.01$ vs. OD) and SOD2 ($p < 0.05$ vs. OD). Selenium per se increased the expression of UCP2 ($p < 0.01$ vs. control) but decreased the expression of SOD2 ($p < 0.01$ vs. control). Combination of Se + HG increased the expression of UCP2 ($p < 0.01$ vs. HG) but decreased the expression of SOD2 ($p < 0.05$ vs. HG) in HG group. Selenium treatment in OD + HG group increased the protein levels of UCP2, and SOD2 to a less extent compared to OD + HG ($p < 0.01$ vs. OD + HG).

Selenium Activates Mitochondrial Biogenesis Signaling After OD an OD + HG in the HT22 Cells

To explore the effects of selenium on mitochondrial biogenesis, we detected key regulators including PGC-1α, NRF1 and TFAM using Western blot (Fig. 3a). The results demonstrated that the levels of PGC-1α, NRF1 and TFAM in HT22 cells decreased mildly after HG exposure (Fig. 3b–d, $p < 0.05$ *vs.* Control). OD increased the levels of PGC-1α, NRF1 and TFAM in cultured HT22 cells compared with control cells (Fig. 3c, d, $p < 0.01$). OD + HG increased these three markers as well ($p < 0.01$ *vs.* Control), but to a lesser extent than in OD (Fig. 3b–d, $p < 0.01$ or $p < 0.05$ vs. OD). Selenium per se matkedly increased the protein levels of PGC-1α, NRF1 and TFAM ($p < 0.01$ vs. Control).

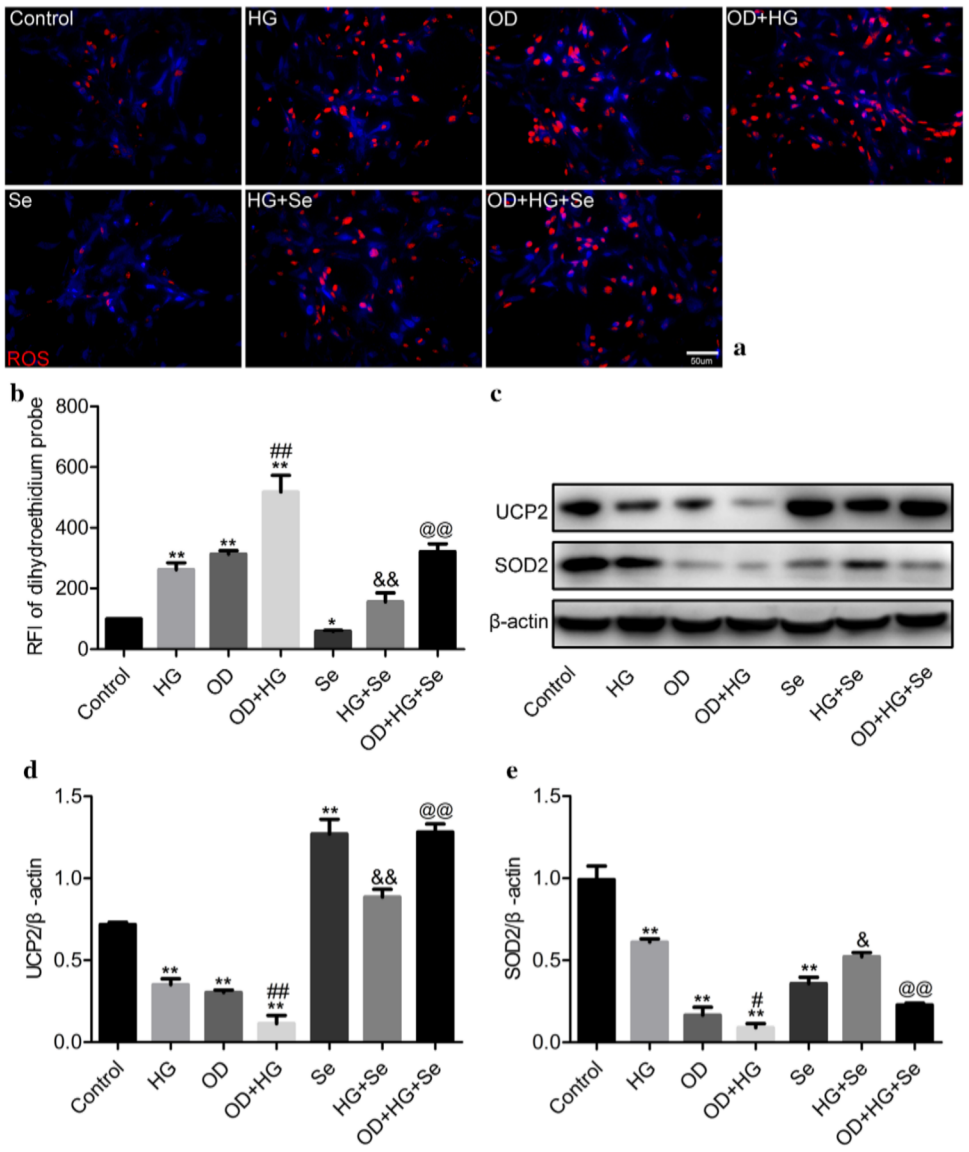

Fig. 2 Effects of selenium on UCP2 and SOD2 protein levels in HT22 cells. **a** Labeling of superoxide by DHE probe. Red color, DHE; Blue color, DAPI. Scale bar = 50 μm. **b** Quantification of the relative DHE fluorescence intensity (RFI). **c** Representative Western blotts of UCP2 and SOD2 in whole cell lysate. **d** Relative protein band density values for UCP2. **e** Relative protein band density values for SOD2. Experiments were repeated 4 times with triplicates for each condition each time. Data are presented as mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. OD group; $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ vs. HG group; $^{@@}p < 0.01$ vs. OD + HG group

Pretreatment with selenium to both HG group ($p < 0.01$ vs. HG) and OD + HG group ($p < 0.01$ vs. OD + HG) markedly increased the levels of PGC-1α, NRF1 and TFAM as well (Fig. 3b–d).

Blood Glucose Concentrations and Body Weights in the Rats

In animal study, the blood glucose levels were significantly increased in the hyperglycemic groups within one week after STZ-injection (Table 2). The increases of blood glucose maintained till 8 weeks after STZ injection. The blood glucose levels in HI (30.3 ± 3.5 mM) and HI + Se (25.8 ± 6.7 mM) groups were significantly higher than that of the NI (5.7 ± 0.6 mM) group ($p < 0.01$). However, there was no statistically significant difference between the HI group and the HI + Se group ($p > 0.05$). The body weights of the NI animals increased steadily every week from 221 in frist week to 374 in 8th week (Table 3). The body weights of the HI animal group stayed at low level of 186–205 g for at least 6 weeks and then increased to 227–240 g in 7th and 8th weeks. The blood glucose levels in the HI group were significant lower than those in NI group ($p < 0.05$). The body weights in HI + Se group had similar trend with HI group. The body weights were significantly lower that those in NI group starting to 3rd week to 8th week. There were no significant differences in body weight between HI and HI + Se groups (Table 3).

Selenium Reduces Infarct Volume, Neurological Deficits and Mortality

Measurements of infarct volume using TTC staining reveal that hyperglycemia enlarged and selenium reduced ischemic

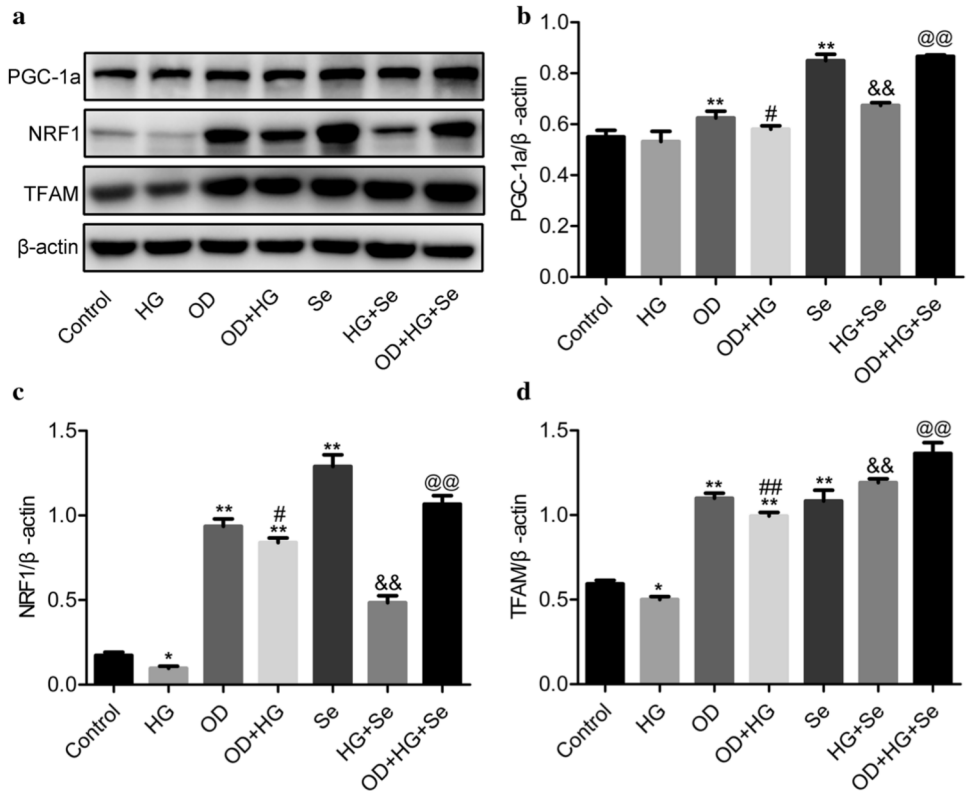

Fig. 3 Effects of selenium on expression of mitochondrial biogenesis markers PGC-1a, NRF1 and TFAM in HT22 cells. **a** Representative Western blotts. **b**–**d** Relative protein band density values of PGC-1α, NRF1 and TFAM, respectively. Experiments were repeated 4 times with triplicates for each condition each time. Data are presented as mean ± SD. *$p < 0.05$ and **$p < 0.01$ *vs.* control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. OD group; $^{\&\&}p < 0.01$ vs. HG group; $^{@@}p < 0.01$ vs. OD + HG group

Table 2 Blood glucose concentrations of the animal groups

Groups	1w	2w	3w	4w	5w	6w	7w	8w
NI (mmol/l)	5.2 ± 0.6	5.7 ± 0.7	5.7 ± 0.7	5.6 ± 0.9	5.8 ± 0.1	5.2 ± 0.6	5.6 ± 0.4	5.7 ± 0.6
HI (mmol/l)	22.5 ± 3.4**	18.8 ± 3.4**	25.4 ± 1.9**	21.7 ± 2.0**	21.4 ± 3.2**	28.1 ± 4.9**	30.7 ± 2.6**	30.3 ± 3.5**
HI + Se (mmol/l)	19.2 ± 1.3**	19.6 ± 3.3**	24.4 ± 4.2**	20.4 ± 1.7**	20.7 ± 1.7**	23.1 ± 4.4**	21.9 ± 4.3**	25.8 ± 6.7**

Data are presented as mean ± SD. N = 10 per group. *$p < 0.05$ and **$p < 0.01$ vs. NI group

NI normoglycemic ischemia, *HI* hyperglycemic ischemia, *Se* sodium selenite, *w* week

Table 3 Body weights of the animal groups

Groups	1w	2w	3w	4w	5w	6w	7w	8w
NI (g)	221.3 ± 24.6	241.8 ± 23.4	265.5 ± 24.6	296.0 ± 24.8	314.5 ± 16.0	329.8 ± 29.7	352.8 ± 21.5	374.3 ± 16.5
HI (g)	202.5 ± 6.8	205.8 ± 4.0	204.0 ± 9.2**	205.3 ± 16.5**	194.5 ± 43.6**	186.8 ± 46.9**	240.0 ± 42.1**	227.5 ± 50.9**
HI + Se (g)	203.5 ± 15.4	212.0 ± 6.7	213.8 ± 29.4*	219.8 ± 33.6**	234.8 ± 46.7*	245.0 ± 49.9*	267.5 ± 57.9*	243.8 ± 71.1**

Data are presented as mean ± SD. N = 10 per group. *$p < 0.05$ and **$p < 0.01$ vs. NI group

NI normoglycemic ischemia, *HI* hyperglycemic ischemia, *Se* sodium selenite, *w* week

infarct volume (Fig. 4a). As expected, 30 min of MCAO in normoglycemic animals resulted in small infarct volumes (3.6%) that were confined the striatum after 24 h of reperfusion. In some case, TTC staining yielded pinkish area, which reflects selective neuronal necrosis. In contrast, 30 min of MCAO in hyperglycemic animals produced a significantly larger infarct volume (44.72%) than in the NI group (Fig. 4b, $p < 0.01$ vs. NI). Further, the damage was also extended to the neocortex. Pretreatment with selenium in HI animals significantly decreased the infarct volume in the hyperglycemic animals to 3.2% (Fig. 4b, $p < 0.01$ vs. HI). Hyperglycemia worsened and selenium improved neurological function (Fig. 4c). Neurobehavioral assessment showed that HI animals had a higher neurological deficit scores than the

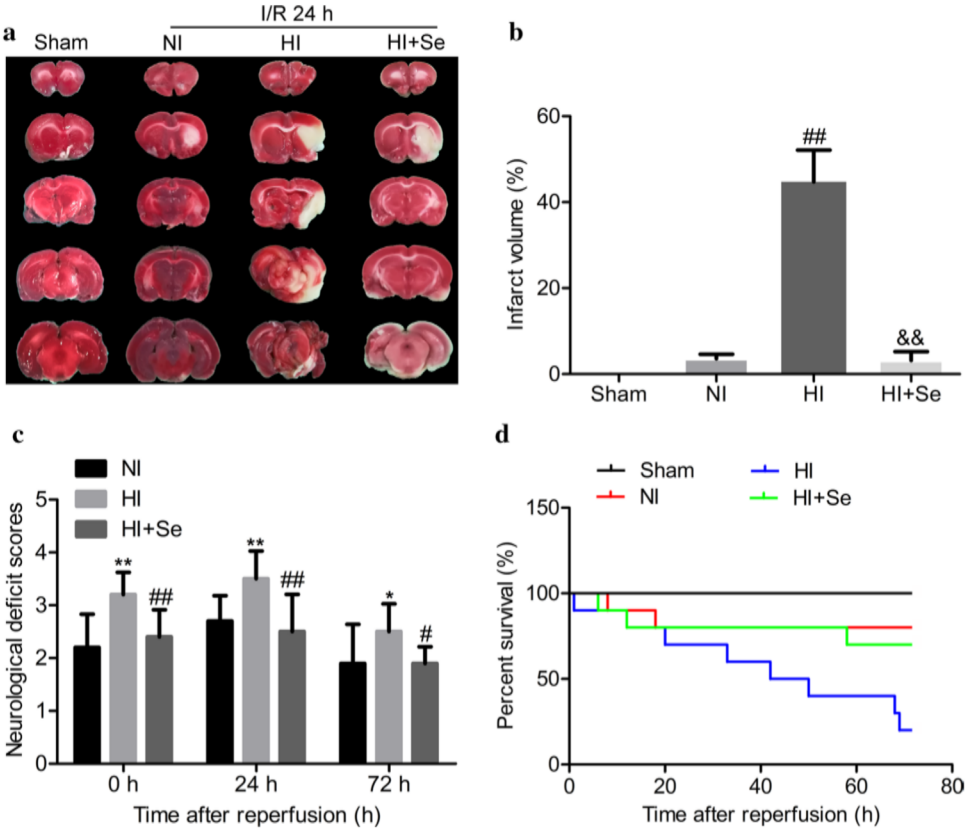

Fig. 4 Effects of selenium on infarct size, neurological scores and mortality rates after MCAO in the rat. **a** Representative TTC-stained sections. Pale areas show infarct area. **b** Bar-graph showing measurements of infarct volumes (n = 4–7 per group). Data are presented as mean ± SD. $^{\#\#}p < 0.01$ vs. NI group; $^{\&\&}p < 0.01$ vs. HI group. **c** Summary of the neurological deficits score (n = 10). $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* respective NI group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. respective HI group. **d** Summary of the survival rate (n = 10 in each group)

NI animals, which was persistent from immediately after MCAO to 72 h after reperfusion. Pretreated with selenium for 4 weeks significantly reduced the neurological deficit scores in the HI animals (Fig. 4c, $p < 0.05$). Again, this decrease was observed immediately after MCAO as well as after 24 h and 72 h of reperfusion. Hyperglycemia increased and selenium decreased the mortality (Fig. 4d). After the induction of I/R, 2 out of 10 animals in NI group died between 8 and 18 h. In contrast, 3 out of 10 HI animals died at 20 h of reperfusion, 2 additional rats at 42 h and another 3 at 72 h died in HI group. Thus, the animal survival rate in HI group reduced to 70% at 20 h, 50% at 42 h and 20% at 72 h, which is significantly lower than that of NI group ($p < 0.01$ vs. NI). Selenium treatment significantly reduced the death rate in HI animals (Fig. 4d). Therefore, only 3 out of 10 animals (30%) died at 72 h of reperfusion ($p < 0.05$ vs. HI).

Selenium Improves Histologic Outcomes

Brain sections were stained by H&E staining and Nissl staining (Fig. 5). To differentiate the damage between NI and HI, we selected to present the data from the fronto-parietal cortex. As shown H&E staining in Fig. 5a, upper panel, several shrunken neurons and vacuoles around the neurons, a presentation of brain edema, were observed at the ischemic region in NI group. The number of neuronal pyknosis and vacuoles in brain tissue was largely increased in the HI group compared to NI group (Fig. 5b, $p < 0.01$), suggesting severe neuronal damage and brain edema. Compared with the HI group, pretreatment of selenium in HI animals reduced the number of neuronal pyknosis and extent of brain edema ($p < 0.01$).

Nissl staining (Fig. 5a lower panel and Fig. 5c) revealed that the number of Nissl bodies in the cytoplasm of neurons was moderately decreased in NI group ($p < 0.01$ vs. sham). The number of Nissl bodies was drastically decreased in HI group ($p < 0.01$ vs. NI). Pretreatment with selenium in HI animals improved the presence of Nissl bodies in the neurons ($p < 0.05$ vs. HI).

Selenium Affacts Antioxidant Enzymes in the Rats

To determine whether selenium affects antioxidant enzymes, we detected UCP2 and SOD2 by Western blot using brain tissues collected from ipsilateral hemisphere (Fig. 6a). Semi-quantification of the targeting immunobands showed that UCP2 protein level decreased mildly after 24 h and 72 h in NI group. Hyperglycemia markedly suppressed UCP2 even without ischemic insult. At 24 h and 72 h, UCP2 remained at low levels, which suggests that the hyperglycemia is the suppression factor. After being treated with selenite, UCP2 levels in both

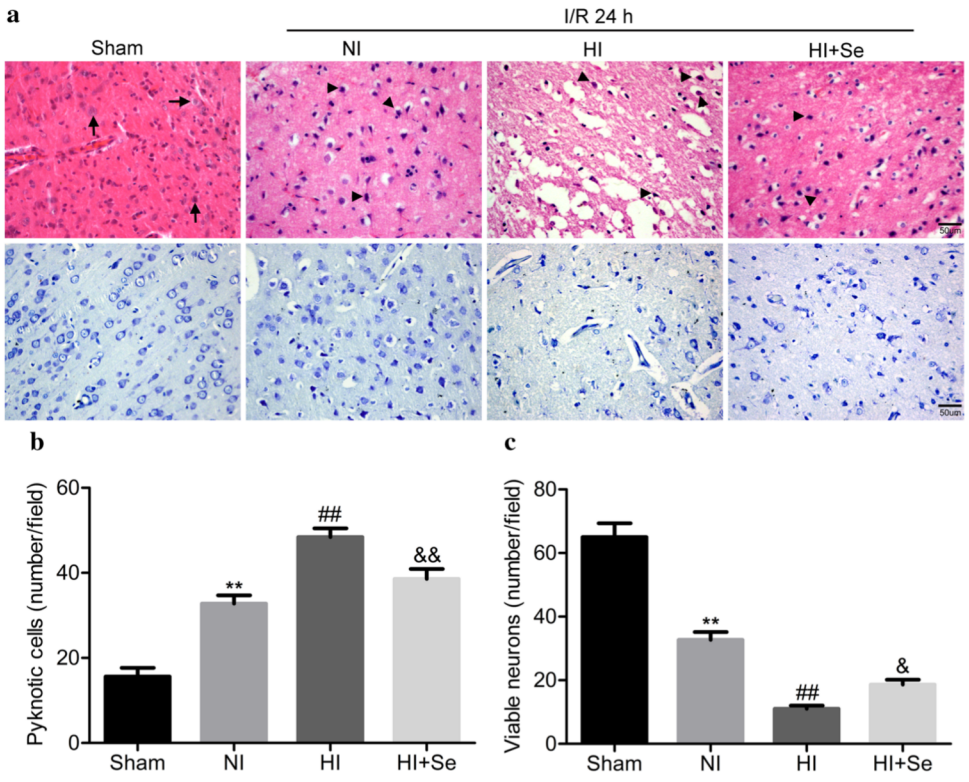

Fig. 5 Histologic outcomes assessed by H&E and Nissl staining after 24 h of reperfusion. **a** Representative images of H&E staining (upper row) and Nissl staining (lower row) from the cortex (400 ×) of the Sham, NI, HI, and HI + Se animls.. Arrows point to normal neurons in the sham group and arrowheads indicate pyknotic and swollen neurons. Scale bar = 50 μm. **b** Quantification of pyknotic cells in the fronto-parietal cortex using H&E stained brain sections. **c** Quantification of the viable neurons in the fronto-parietal cortex using Nissl staining. Data are presented as mean ± SD. N = 5–8 per group. $^{**}p < 0.01$ vs. sham group; $^{\#\#}p < 0.01$ vs. NI group; $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ vs. HI group

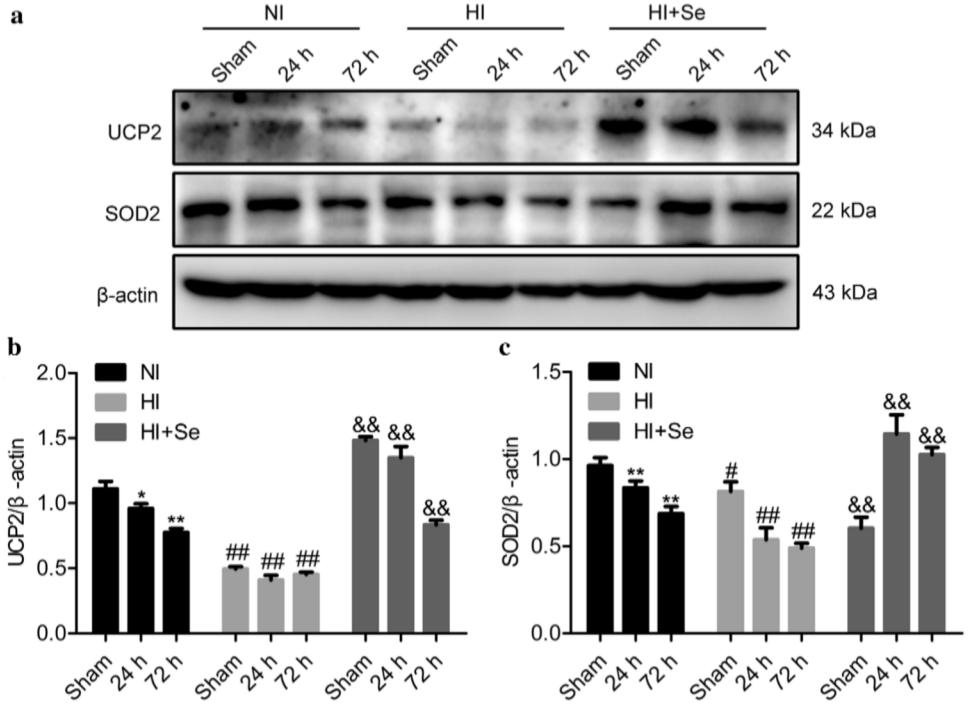

Fig. 6 UCP2 and SOD2 protein levels in rat cortical area at 24 or 72 h I/R. **a** Representative Western blotts of UCP2 and SOD2. **b**, **c** Relative protein band density ratios of UCP2 or SOD2 to β-actin. Data are presented as mean ± SD. N = 3–4 per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. respective sham-operated controls; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. NI counterpart at an identical reperfusion stage; $^{\&\&}p < 0.01$ vs. HI counterpart at an identical reperfusion stage

hyperglycemic sham and HI groups increased drastically when compared to normoglycemic and hyperglycemic groups (Fig. 6b). Changes of SOD2 followed the same pattern. Therefore, ischemia in normoglycemic animals caused mild to moderate decreases at 24 h and 72 h. Hyperglycemia led to more pronounced declines at 24 h and 72 h compared with NI animals ($p < 0.01$). Selenium treatment in hyperglycemic animals increased SOD2 after ischemia at 24 h and 72 h (Fig. 6c, $p < 0.01$ vs. HI).

Selenium Regulates Mitochondrial Biogenesis Pathway in the Rats

TEM examination showed that round nucleus, intact nuclear membrane, clear nucleolus and abundant cytoplasm could be observed in the sham-operated animals. Part of nuclear pyknosis, bending of nuclear membrane and reduced cytoplasm could be observed in the NI group. Nuclear pyknosis was more prominent in the HI group, while selenium treatment improved nuclear pyknosis in HI group compared the HI group (Fig. 7a, upper panel). Further, a few damaged mitochondria with disrupted or broken cristae were observed in the NI group (arrowhead in Fig. 7a, lower panel) compared with the sham group. In the HI group, the richness of mitochondria in the microscopic field seemed decreased. The mitochondrial swelling became more severe and the mitochondrial crest was difficult to identify compared with sham and NI animals (arrowheads in Fig. 7a, lower panel). Selenium treatment in HI animals reinstalled the mitochondrial density in the cytoplasm, reduced mitochondrial swelling and improved the integrity of mitochondrial cristae (arrows in Fig. 7a lower panel).

To investigate whether ischemia under normo- and hyperglycemic conditions affects mitochondrial biogenesis signaling and whether selenium is able to prevent the modulating effects of ischemia on mitochondrial biogenesis,

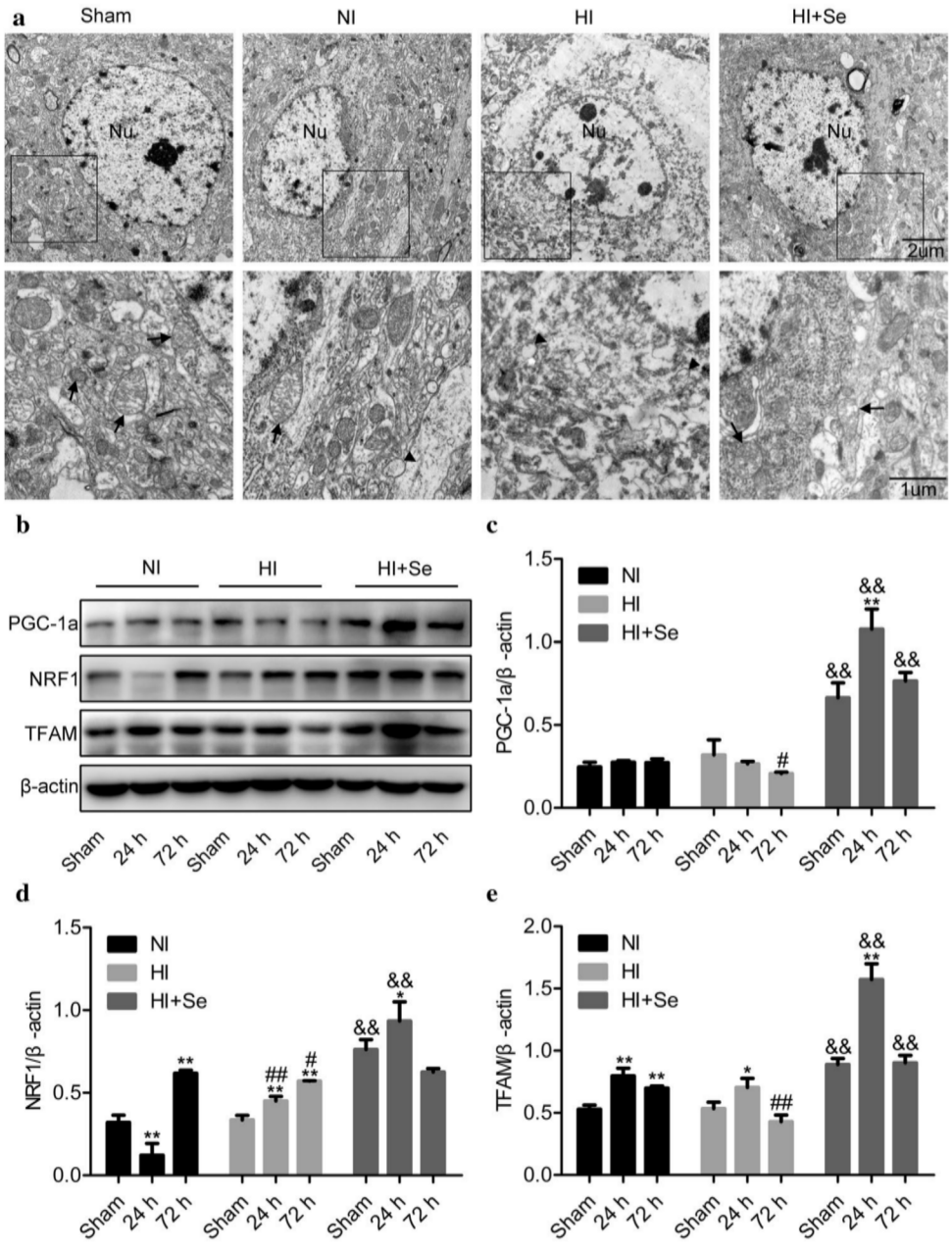

Fig. 7 Changes of mitochondrial ultrastructure and biogenesis markers in rat cortical area at 24 h or 72 h I/R. **a** Representative TEM micrographs from cortical neurons in NI, HI, and HI + Se treated animals subjected to 30 min MCAO and 24 h of reperfusion. The bottom row is the enlargement of the contents of the black box on the top row. Arrows indicate normal mitochondria and arrowheads point to damaged mitochondria. Scale bar = 2 μm for upper row and 1 μm for lower row. Nu = neuronal nucleus. **b** Representative Western blotts of mitochondrial biogenesis markers PGC-1a, NRF1 and TFAM in cortical area at 24 or 72 h I/R. **c–e**, Relative target protein band density ratios of PGC-1a, NRF1 or TFAM to β-actin, respectively. Data are presented as mean ± SD. N = 3–4 per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. respective sham-operated controls; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. NI counterpart at an identical reperfusion stage; $^{\&\&}p < 0.01$ vs. HI counterpart at an identical reperfusion stage

we measured the protein levels of 3 factors that are known to regulate mitochondrial biogenesis, PGC-1α, NRF1 and TFAM in brain tissues collected from ipsilateral hemisphere (Fig. 7b). Western blot showed that PGC-1α was not changed in NI, but decreased at 72 h of reperfusion in HI animals ($p < 0.05$ vs. NI). Selenium resulted in pronounced increases of PGC-1α with or without ischemia in hyperglycemic animals (Fig. 7c, $p < 0.01$). NI caused a biphasic change in NRF1, an initial decrease at 24 h and followed by an increase at 72 h of reperfusion ($p < 0.01$ vs. sham). HI increased the NRF1 after 24 h and 72 h ($p < 0.01$ vs. sham). However, NRF1 content was lower in HI group at 72 h than that in NI at 72 h ($p < 0.05$). Selenium intervention in hyperglycemic animals significantly increased NRF1 levels under both sham and ischemic conditions (Fig. 7d, $p < 0.01$). NI moderately increased TFAM at 24 h and then started to decline towards control level at 72 h of reperfusion. HI elevated TFAM at 24 h and declined below control value at 72 h. TFAM moderately decreased at 72 h of reperfusion in HI animals than NI animals ($p < 0.01$). Selenium again significantly increased TFAM levels under hyperglycemic sham and hyperglycemic ischemic conditions compared with NI and HI groups (Fig. 7e, $p < 0.01$).

Discussion

Hyperglycemia is well known to exacerbate neuronal injury and infarct volume resulted from cerebral ischemia, and to induce the poor clinical outcomes [15, 16, 18]. In the present study, in vitro OD + HG experiments showed that OD + HG decreased cell viability and increased TUNEL positively stained cells of the HT22 cells. It is interesting that high glucose per se in cell culture decreased cell viability and significantly increase TUNEL positive cells. This suggests that HG itself is harmful to cell survival, which is consistent to increased ROS and reduced levels of UCP2 and SOD2 under high glucose condition. The in vitro results were further conformed by the in vivo experiment showing increased infarct volume, worsend neurological deficit score and decreased survival rate in HI compared with NI rats. These results were consistent with the previous studies and further confirmed that hyperglycemia could aggravate ischemic brain injury [15, 16, 18, 19]. The small infarct volume observed in this experiment is due to the nature of short period of MCAO. 30 min MCAO causes a relative small infarct volume confined to the striatum and the surunding areas have different extent of selective neuronal necrosis [16].

Selenium is an essential micronutrient for human being. It exerts its function through cooporating itself to selenocysteine (Sec), the latter constitutes various selenoproteins [20]. Selenium supplementation boosts immune response and promote human health by promoting the proliferation of activated T cells and increasing tumor cytotoxicity and natural killer cell activity and increasing antioxidant capacity [21]. Selenoproteins prevent the oxidative modification of lipids, reduce platelet aggregation and inhibit inflammation [8]. Deficiency of selenium in human body is associated with development of many diseases, such as osteoarthrosis, Keshan disease, immunodeficiency disease, cardiomyopathy and hypothyroidism [22].

Selenium and seenoproteins have been known for their antioxidant nature [23]. Selenium has been shown to have the ability of protecting against neurodegeneration during ischemia [20]. The administration of selenium enhances learning and memory function in Alzheimer's disease rat model [23]. Selenium increases the content of DNA and expression of mitochondrial proteins in hepatic tissue [24]. However, it is not known whether selenium could alleviate hyperglycemia-aggravated ischemic brain damage. In the present study, we demonstrated that selenium pretreatment protected the HT22 cells from OD + HG-induced decreases of cell viability and increases of TUNEL positively stained cells. The experiment of the hyperglycemic ischemic rats with selenium treatment further conformed the in vitro results. Therefore, the selenium treated HI rats had a longer survival time and better survival rate than the non-selenite treated HI animals. After 30 min of brain ischemia, pretreatment with selenium significantly decreased the infarct volume, decreased neurological deficit scores, and reduced number of degenerative neurons and brain edema. These results suggest that selenium is capable of alleviating hyperglycemia-enhanced hypoxic/ischemic damage both in vitro and in vivo.

Published papers have suggested that hyperglycemia exacerbates cerebral ischemic injury by inducing lactate acidosis, disturbing calcium homeostasis, enhancing free radicals production, increasing the permeability of blood–brain barrier, damaging the structure of mitochondria, and promoting inflammatory response [19]. In the present study, ROS increased in OD and OD + HG further increased the production compared to OD, suggesting that high glucose exacerbates ROS accumulation. Treating HT22 cells with selenium successfully blocked the OD + HG-induced ROS production. The ROS are mainly produced through the mitochondrial electron transportation [25]. In normal conditions, the excessive superoxide radicals produced in the mitochondria is catalized to hydrogen peroxide by SOD2 and the H2O2 is further reduced to carbone dioxide and water by glutathione peroxidase (GPx) or peroxidoxin in the brain [26, 34]. Under pathological conditions, environmental stressors, including inflammatory, metabolic depression, glucose/oxygen deprivation and release of excitatory neurotransmitters, could overwelme the intrinsic antioxidant mechinary and induce oxidative stress [27–30]. Excessive ROS can destroy a series of cellular components including

DNA, proteins and lipids [29]. In the present study, we have demonstrated that high glucose per se inhibited the levels of UCP2 and SOD2. This may explain why high glucose per se is toxic to neurons. The results also demonstrated that OD + HG further reduced the levels of UCP2 and SOD2 comapred with the already low levels of these proteins under OD condition. This may be part of reason accountable for the detrimental effect of hyperglycemia on hypoxic/ischemic damage. We have previously shown that mutation in SOD2 further increses the ischemic brain damage in hyperglycemic animals [35]. Our results are consistent with previous reports showing that UCP2 reduces while hyperglycemia enhances ROS production, and cerebral ischemia inhibits the SOD2 and hyperglycemia enhances ROS [31–33]. In the present study, selenium per se significantly increased the UCP2 level, which is in agreement with the observation that selenium improved cell viability under normal cultures. However, it not clear why SOD2 lelve in selenium per se group was much lower compared with the control. Combinations of HG + Se or OD + HG + Se significantly elevated the level of UCP2, and SOD2 to less extent. Animal study showed that NI reduced the UCP2 and SOD2 levels at 24 h and 72 h of reperfusion following a 30 min MCAO. Hyperglycemia further suppressed the levels of UCP2 and SOD2, even in control animals, which is in agreement with the glucose toxicity to nerons and increased levels of ROS observed in vitro under HG condition. Excessive superoxide production may comsumed large amount of SOD2 beyond being replenished. Selenium treatment to control or HI animals drastically increased the levels of UCP2 and and SOD2. Taken together, our results suggest that selenium reduces the cytotoxicity of glucose and ischemia both in vitro and in vivo through uregulating antioxidant enzymes and this protective effect is associated with increasing UCP2 and SOD2 lelvels.

Activation of mitochondrial biogenesis has been reported in cancer, metabolic diseases, diabetes and human immunedeficiency syndrome [36]. Several reports have suggested that cerebral ischemia may activate or suppress mitochondrial biogenesis [37, 38]. Activation of mitochondrial biogenesis is associated with neuroprotection [37, 39]. Nanjaiah et al. have reported that hyperglycemia reduces mitochondrial biogenesis in retina by down-regulating the expression of PGC-1a and TFAM [40]. Selenium has been shown to activate mitochondrial biogenesis pathway in cultured cells and in ischemic brain [10, 22, 41]. However, it is not known whether selenium is capable of activating mitochondrial biogenesis signaling in hyperglycemic ischemic animals. In the present study, we found that HG per se in cultured cells mildly decreased the levels of PGC-1α, NRF1 and TFAM. OD increased their levels and this could be explained by a normal tissue response to the injury. OD + HG decreased the protein levels of these 3 factors compared with OD. Selenium per se or incombination with HG or OD + HG dramatically increased the protein levels of PGC-1α, NRF1 and TFAM. These results suggest that seleniu is capapble of activating mitochondrial biogenesis pathway in normal neuronal cells and under high glucose and/or OD conditions.The experimental results of animal study were consistent with those in vitro. In geneal ischemia increased the levels of mitochondrial biogenesis markers, hyperglycemia reduced levels compared with NI, and selenium significantly increase the all three markers in HI animals. Specifically, NI increased NRF1 and TFAM but not PGC-1α. This may be because the PGC-1α was upregulated at mRNA level or there are other factor could activate NRF1 and TFAM as well. Ther eresults from NI is consistent with the reports from Dr. Chen's group [37, 38]. Comparired with NI, HI suppressed the protein expression of the three markers at 24 h or 72 h. Administration of selenium to sham operated control or HI animals significantly increased the PGC-1α, NRF1 and TFAM expression levels. Mitochondrial ultrastructual examination demonstrated increased mitochondrial damage in HI compared with NI animals and selenium prevented the damage in HI animals. These data suggest that selenium is capable of activating mitochondrial biogenesis pathway and protect the integrity of mitochondrial structure. Such effects may contribute to its neuroprotective effect against ischemic brain damage in hyperglycemic animals. Therefore, selenium could be used as an important complementary medicine for preventing and treating stroke and other oxidative stress involved disorders.

In summary, both in vitro and in vivo models of hyperglycemic hypoxia/ischemia have demonstrated that hyperglycemia enhances neuronal damage and such adverse effect of glucose is associated with increased ROS production and suppressed UCP2 and SOD2. Treatment with sodium selenite reduced the damage, increased antioxidant enzymes and reduced ROS content in hyperglycemic ischemic animals. In addition, selenium activates mitochondrial biogenesis signaling in hyperglycemic ischemic animals.

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Compliance with Ethical Standards

Conflict of interest The authors have declared that no competing interest exists.

Ethical Approval All animal procedures were approved by Institutional Animal Care and Use Committee (IACUC) at the Ningxia Medical University.

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