Time-dependent homeostasis between glucose uptake and consumption in astrocytes exposed to $CoCl_2$ treatment

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Abstract. Hypoxia has been implicated in the pathology of the central nervous system during stroke. It also has a significant effect on the regulation of glucose transporters (GLUTs), and homeostasis between glucose uptake and consumption. $CoCl_2$ is a hypoxia-mimetic agent, and thus stabilizes the hypoxia-inducible factor 1α (HIF-1α) subunit and regulates GLUT genes. GLUT-1 and GLUT-3 are the most common isoforms of the GLUT family present in the brain, with the former primarily expressed in astrocytes and the latter in neurons under physiological conditions. However, it remains controversial whether GLUT-3 is expressed in astrocytes. Additionally, it is unclear whether the regulation of GLUT-1 and GLUT-3, and glucose homeostasis, are affected by $CoCl_2$ treatment in a time-dependent manner. In the present study, mRNA and protein levels of GLUT-1, GLUT-3 and HIF-1α in astrocytes were examined by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. The intracellular glucose concentration, glycogen storage, ATP content, pyruvate concentration, lactate dehydrogenase (LDH) release activity and cell viability in astrocytes were also investigated. The observations of the current study confirmed that both protein and mRNA levels of GLUT-1 and GLUT-3 were elevated in a time-dependent manner induced by $CoCl_2$ treatment, followed by accumulation of HIF-1α. Furthermore, in the early period of $CoCl_2$ treatment (≤8 h at 100 μM), LDH release, ATP content, glycogen storage and cell viability remained unchanged, whereas intracellular pyruvate concentration increased and glucose concentration was reduced. However, in the later period of $CoCl_2$ treatment (>8 h at 100 μM), LDH release and intracellular pyruvate concentration increased, while intracellular glucose concentration, ATP content and glycogen storage were reduced. This may be due to disruption of homeostasis and reduced cell viability. In conclusion, alteration in the expression levels of GLUT-1 and GLUT-3, and the homeostasis between glucose uptake and consumption were affected by $CoCl_2$ treatment, in a time-dependent manner, and may result in reduced energy production and cell viability in astrocytes.

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Introduction

Stroke continues to be a leading worldwide cause of human mortality and long-term disability, with ~70% of stroke survivors experiencing reduced work capacity and ≤30% requiring self-care assistance (1). A reduction or complete blockage of blood flow to regions of the brain results in oxygen and glucose deficiencies, which may lead to ischemic stroke (2,3). The weight of the brain is 2% of the total body weight; however, it uses nearly 20% of the body's cardiac output to achieve its supply of essential nutrients, including oxygen and glucose (4). Glucose is the primary source of energy that sustains cellular activity and homeostasis in the brain. Under the conditions of a stroke, more glucose is required due to the rapid depletion of oxygen from compensatory metabolic alterations (5). Severe reduction in the supply of glucose and oxygen to the brain, even for a short period of time, often initiates brain ischemia-reperfusion, which leads to a complex cascade of cellular events, resulting in neuronal death and, consequently, loss of brain function (6).

Glucose transporters (GLUTs) are present in all types of cells and are responsible for the entry of glucose into cells without consuming energy throughout the periphery and the brain. Therefore, the expression, regulation and activity of GLUTs are important for neural homeostasis (7). Additionally, the enhanced activity of GLUTs protects cells during energy depletion under hypoxic conditions (8). Hypoxia-inducible factor 1α (HIF-1α), an important regulator of the cellular response to oxygen deprivation (9), is a key transcription factor for various genes involved in glucose uptake, angiogenesis, glycolysis, pH balance and metastasis (10). In a hypoxic

environment, the HIF-1α signaling pathway is activated and, in turn, GLUTs and glycolytic enzymes are activated, promoting glucose uptake in cells through the transcription of GLUTs (11) to maintain adenosine triphosphate (ATP) levels essential for cell survival (12,13).

Astrocytes are star-shaped glial cells of the central nervous system, and provide an important link between endothelial cells and neurons (14). The regulation of glucose uptake in astrocytes is important for normal brain function because glucose taken up by astrocytes is also used to supply neurons with metabolic substrates required to sustain neuronal functions, such as synaptic transmission (10,15). Although numerous isoforms of GLUTs have been identified in the brain, GLUT-1 and GLUT-3 are the most abundant (16,17). Under physiological conditions, GLUT-1 is primarily expressed in neurons and at the plasma membranes in astrocytes; however, controversy remains as to whether GLUT-3 is also expressed in astrocytes (16,18). A previous study indicated that GLUT-3 was not expressed in astrocytes but in neurons (19); however, other studies have detected GLUT-3 at extremely low levels in cultured astrocytes under physiological conditions (10,18).

Numerous physiological pathways, each with a distinctive time frame, are spontaneously activated following the onset of stroke (20). It is unclear whether the regulation of GLUT-1, GLUT-3 and glucose homeostasis behave in a time-dependent manner under hypoxic conditions. $CoCl_2$ is a hypoxia mimetic agent that may produce a hypoxic-like environment and activate HIF-1α under normoxic conditions *in vitro* and *in vivo* (13,21). $CoCl_2$ exposure also leads to mitochondrial damage and increases the generation of reactive oxygen species (20,22). These observations suggest that a model of hypoxic damage induced by $CoCl_2$ is a suitable tool to investigate the mechanisms of cell injury (23). Furthermore, hypoxia and $CoCl_2$ exposure were associated with compromised ATP production, possibly associated with metabolic alterations, which resulted in death of the astrocytes (24). Therefore, the current study was undertaken to investigate the regulation of GLUTs and glucose homeostasis including glucose uptake and consumption during $CoCl_2$ treatment, which may suggest an optimal time frame following stroke to commence with therapy. Further understanding of hypoxia-induced alterations in the homeostasis of glucose uptake and consumption would facilitate the design of effective rehabilitative strategies.

Materials and methods

Materials. All cell culture reagents, including media, antibiotics, fetal bovine serum (FBS) and phosphate-buffered saline (PBS), were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Mouse monoclonal anti-GLUT-1, rabbit monoclonal anti-GLUT-3, mouse monoclonal anti-HIF-1α and mouse monoclonal anti-β-actin were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Cell culture plastics were purchased from Corning Incorporated (Corning, NY, USA). TRIzol reagent, SuperScript III reverse transcriptase and Platinum SYBR-Green qPCR SuperMix were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). A cytotoxicity detection kit was purchased from Roche Diagnostics (Indianapolis, IN, USA) and an Amplex Red Glucose Assay kit was purchased from Life Technologies (Thermo Fisher Scientific, Inc). An ATP assay kit was purchased from Merck Millipore (Darmstadt, Germany). The pyruvate assay kit and the glycogen assay kit were purchased from BioVision, Inc. (Milpitas, CA, USA). The remaining chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals. A total of 22, 1-day-old BALB/c mice, weighing 2±0.3 g were obtained from the Experimental Animal Centre of Zhengzhou University (Zhengzhou, China), housed in a pathogen-free environment, maintained on a 12-h light-dark cycle and fed with commercial pellets. All experiments were approved by the Ethics Committee of Life Sciences, Zhengzhou University (Zhengzhou, China).

Primary astrocyte culture. The primary astrocyte culture was prepared as described previously (22). Briefly, the neonatal mice were sacrificed by decapitation following anesthesia with 30 mg/kg Zoletil 100 (Virbac Laboratories, Carros, France), cerebral cortices were removed, and tissue was transferred to complete Dulbecco's modified Eagle's medium (DMEM; supplemented with 10% FBS, 25 mM glucose, 50 U/ml penicillin and 50 mg/ml streptomycin-sulfate) and dissociated with 0.0025% trypsin/ethylenediaminetetraacetic acid. Cells were seeded at a density of $3x10^4$ cells/cm^2 in complete DMEM at 37˚C in a humidified atmosphere with 5% CO_2. The medium was renewed on day *in vitro* (DIV) 1, DIV 5 and DIV 7. On DIV 9, microglia were discarded using the shake-off method (23). Astrocytes were harvested with trypsin and reseeded at a density of $3x10^4$ cells/cm^2 in complete DMEM/F12 medium. The homogeneity of astrocytes was 90-95% by detection of glial fibrillary acidic protein with 5-10% of cells being B4 isolectin$^+$ microglia. Astrocytes were reseeded at a density of $5x10^4$ cells/cm^2 and incubated at 37˚C with 5% CO_2. Experiments were performed on cells 10-18 days after plating at which point they formed an incomplete monolayer of stellate- and flat-shaped astrocytes. cells were exposed to 100 μM $CoCl_2$ and tested at distinct time points.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To examine GLUT-1 and GLUT-3 gene expression induced by $CoCl_2$ treatment according to the time courses, astrocytes were harvested following incubation with 100 μM $CoCl_2$ at different time points (0, 2, 4, 6, 8, 10, 12, 18 and 24 h). Total RNA was extracted by using TRIzol reagent, according to the manufacturer's protocol, and subjected to DNase treatment using a FastQuant RT kit (Tiangen Biotech Co., Ltd., Beijing, China). RNA quantity and quality was determined spectrophotometrically at 260 and 280 nm using an EU-2200R Ultraviolet spectrophotometer (Shanghai Onlab Instruments Co., Ltd., Shanghai, China). Reverse transcription of 20 ng RNA was performed using a SuperScript III reverse transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.) in preparation for qPCR.

GLUT-1, GLUT-3, HIF-1α and β-actin expression levels were evaluated by qPCR using Platinum SYBR-Green qPCR SuperMix. The running conditions were 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles at 95˚C for

Table I. Specific primers for the expression of GLUT-1, GLUT-3, HIF-1α and β-actin.

Gene name	Primer sequence (5'-3')	Length, bp	Amplicon, nt
GLUT-1	F: GACCCTGCACCTCATTGG	18	106
	R: GATGCTCAGATAGGACATCCAAG	23	
GLUT-3	F: GGAGGAAGACCAAGCTACAGAG	22	130
	R: GAGCTCCAGCACAGTCACCT	20	
HIF-1α	F:AACAGAATGGAACGGAGCAA	20	119
	R: TTCACAATCGTAACTGGTCAGC	22	
β-actin	F: CTAAGGCCAACCGTGAAAAG	20	104
	R: ACCAGAGGCATACAGGGACA	20	

GLUT-1/3, glucose transporter-1/3; HIF-1α, hypoxia-inducible factor-1α; F, forward; R, reverse; bp, base pairs; nt, nucleotides.

15 sec and 60˚C for 1 min. qPCR was performed using the ABI 7500 sequence detector (Applied Biosystems; Thermo Fisher Scientific, Inc.). The design of the specific primers is presented in Table I.

The quantities of gene-specific mRNA expression were determined by the quantification cycle (Cq) method and the Cq value for β-actin was used as an internal calibrator. The comparative Cq method, or $2^{-\Delta\Delta Cq}$, was used for relative quantization (25) using the following equation: $^{\Delta\Delta}Cq = (Cq_{target} - Cq_{calibrator})_{experimental} - (Ct_{target} - Cq_{calibrator})_{control}$. Data are presented as ratios over control (time point, 0 h).

Western blot analysis. The astrocytes were exposed to 100 μM $CoCl_2$ for the aforementioned periods of time, then the cells were collected and solubilized in lysis buffer (60 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM KCl, 5 mM Na_3 EDTA, 3 mM EGTA, and 1% Triton X-100) with protease inhibitors. Following centrifugation at 14,000 x g for 15 min at 4˚C, the supernatant was collected and solubilized in loading buffer. Samples were normalized to protein concentration and proteins were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis with a running voltage of 200 v for 30 min and electrophoretically transferred to polyvinylidene difluoride membranes at 100 V for 1 h. The membranes were blocked with 5.0% fat-free milk in PBS with 0.05% Tween-20 (PBST) for 1 h at room temperature, and incubated separately with mouse monoclonal anti-GLUT-1 (cat. no. sc-377228; dilution 1:1,000), rabbit GLUT-3 (cat. no. sc-74399; dilution 1:1,000), mouse monoclonal anti-HIF-1α (cat. no. sc-71247; dilution 1:1,000) or mouse β-actin (cat. no. sc-47778; dilution 1:2,000; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. Subsequently, the membranes were washed with PBST three times for 10 min each. Following incubation with Alexa Fluor® 488 donkey anti-rabbit IgG (cat. no. R37118) and Alexa Fluor® 680 rabbit anti-mouse IgG (cat. no. A-21065; Thermo Fisher Scientific, Inc.; dilution 1:10,000) for 60 min at room temperature in the dark, blots were detected on an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and relative density units were estimated from the mean pixel density using Image Studio Lite version 4.0 (LI-COR Biosciences) and normalized to β-actin. Data are presented as ratios over control (time point: 0 h).

Measuring intracellular glucose concentration. Astrocytes were seeded at a density of $5x10^4$ cells/cm^2 in a 12 well plate and exposed to 100 μM $CoCl_2$ for the aforementioned time periods when cells were confluent. In the final 2 h of each time course, astrocytes were incubated in glucose-free medium (25 mM mannose) for 1 h, and then in 25 mM glucose medium for 1 h. During the incubation in medium containing 25 mM mannose and 25 mM glucose, astrocytes of the $CoCl_2$ treatment group were still treated with 100 μM $CoCl_2$. Subsequently, cells were rapidly chilled on ice and washed four times with ice-cold PBS, then they were collected and lysed in 500 μl of ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1 mM benzamidine, 0.5% Triton X-100) with protease inhibitors (Sigma-Aldrich). The intracellular glucose concentration in cell lysates was determined using the Amplex Red Glucose Assay kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Subsequently, 50 μl cell lysate was mixed with 50 μl reaction mixture and incubated for 30 min at room temperature in the dark. The absorbance was then measured at 560 nm using an SP-Max 2300A2 microplate reader (Shanghai Flash Spectrum Biological Technology Co., Ltd., Shanghai, China). Intracellular glucose concentration was determined from a standard curve generated using the various concentrations normalized to the protein concentration. Data are presented as the percentage of glucose concentration compared with control (time point of $CoCl_2$ treatment, 0 h), which is expressed as 100%.

Quantification of intracellular glycogen. Total glycogen concentration was determined using a colorimetric glycogen assay kit (BioVision, Inc.), according to the manufacturer's protocol. The astrocytes were homogenized with dH_2O on ice and the homogenates were boiled for 5 min to inactivate the enzymes present. The boiled samples were spun at 15,000 x g at 4˚C for 5 min to remove insoluble material, then the supernatant was collected for assaying optical density, which was measured at 570 nm using a microplate reader (SP-Max 2300A2; Shanghai Flash Spectrum Biological Technology Co., Ltd.). Glycogen concentration was determined from a standard curve generated using various concentrations of glycogen and normalized to protein concentration. Data are presented as the percentage glycogen concentration compared with control (time point, 0 h), which is expressed as 100%.

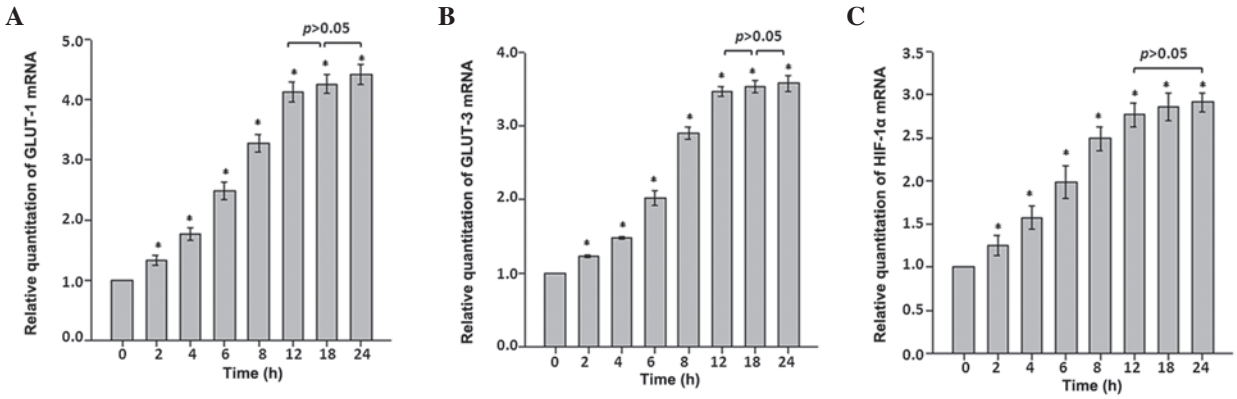

Figure 1. Effects of $CoCl_2$ treatment on mRNA expression levels of GLUT-1, GLUT-3 and HIF-1α. $CoCl_2$ treatment led to increased mRNA expression levels of (A) GLUT-1, (B) GLUT-3 and (C) HIF-1α. Cq values were used to determine the quantities of gene-specific mRNA expression, and the $2^{-\Delta\Delta Cq}$ method was used for relative quantitation. β-actin was used as an internal calibrator. Values were presented as ratios over control (0 h). Data is presented as the mean ± standard deviation of three independent experiments and samples were analyzed in triplicate (n=9). *P<0.05 vs. control. GLUT-1/3, glucose transporter-1/3; HIF-α, hypoxia-inducible factor-1α.

Measuring intracellular pyruvate. Pyruvate was extracted from the astrocytes using 4 volumes of the pyruvate assay buffer. The cells were centrifuged (10,000 x g; 10 min; 4˚C) to remove insoluble material and collect the supernatant. A total of 2-50 µl supernatant was added into a 96-well plate and the volume adjusted to 50 µl/well with pyruvate assay buffer. Next, the 50 µl reaction mixture (containing 46 µl pyruvate assay buffer, 2 µl pyruvate probe and 2 µl enzyme mix) was added. Following incubation for 30 min at room temperature in the dark, intracellular pyruvate was measured by colorimetric assay using a pyruvate assay kit (BioVision, Inc.). Optical density was measured at 570 nm using a microplate reader (SP-Max 2300A2; Shanghai Flash Spectrum Biological Technology Co., Ltd.). Intracellular pyruvate was determined from a standard curve generated using various concentrations of pyruvate and normalized to protein concentration. Data are presented as the percentage pyruvate concentration compared with the control (time point of $CoCl_2$ treatment=0 h), which is expressed as 100%.

Measuring intracellular ATP content. Astrocytes were seeded at a density of $5x10^4$ cells/cm^2 in a 12-well plate and were exposed to 100 µM $CoCl_2$ for testing at distinct time points. Cells of the control group were generated at the same time points without $CoCl_2$ treatment. Following the aforementioned cell preparations, intracellular ATP content was determined by bioluminescence assay using a commercial ATP assay kit (Merck Millipore), according to the manufacturer's protocol. The astrocytes were treated with 100 µl nuclear releasing reagent for 5 min at room temperature while gently shaking, then 1 µl ATP monitoring enzyme was added to the cell lysate. Bioluminescence of intracellular ATP content was determined from a standard curve generated using various concentrations of ATP and normalized to protein concentration.

Measuring cell viability. Astrocyte cell viability was demonstrated at distinct time points following $CoCl_2$ treatment using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, according to the manufacturer's protocol. Astrocytes of the control group were cultured without $CoCl_2$ treatment and generated at the same time points. MTT solution (5.0 mg/ml) was added to the cells and incubated at 37˚C for 4 h. The culture medium was aspirated, and 1 ml dimethyl sulfoxide was added and thoroughly mixed for 10 min. MTT absorbance was measured at 570 and 630 nm using a microplate reader (SP-Max 2300A2; Shanghai Flash Spectrum Biological Technology Co., Ltd.).

Measuring lactate dehydrogenase release. To measure lactate dehydrogenase (LDH) release in the astrocyte supernatant, the supernatant was collected by centrifugation at 250 x g at 4˚C for 10 min and transferred into a 96-well plate following exposure to $CoCl_2$ treatment for the indicated time points. Astrocytes of the control group were cultured without $CoCl_2$ treatment and the supernatant was generated at the same time points. LDH was determined using a cytotoxicity detection kit (Roche Diagnostics) according to the manufacturer's protocol, with Triton X-100 (2.0%) serving as a positive control. Absorbance was measured using a microplate reader (SP-Max 2300A2; Shanghai Flash Spectrum Biological Technology Co., Ltd.) at a test wavelength of 490 nm and a reference wavelength of 630 nm.

Statistical analysis. Statistical analyses was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Data from multiple experiments were processed and are expressed as the mean ± standard deviation. Statistical differences between the different time points were determined using one-way analysis of variance followed by a Student-Newman-Keuls test. Statistical analyses of intracellular ATP content, cell viability and LDH release at all time points between two groups were performed using the independent-samples t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of $CoCl_2$ treatment on mRNA expression levels of GLUT-1, GLUT-3 and HIF-1α. The time course analysis using RT-qPCR indicated that GLUT-1 and GLUT-3 mRNA expression levels in astrocytes changed in a time-dependent

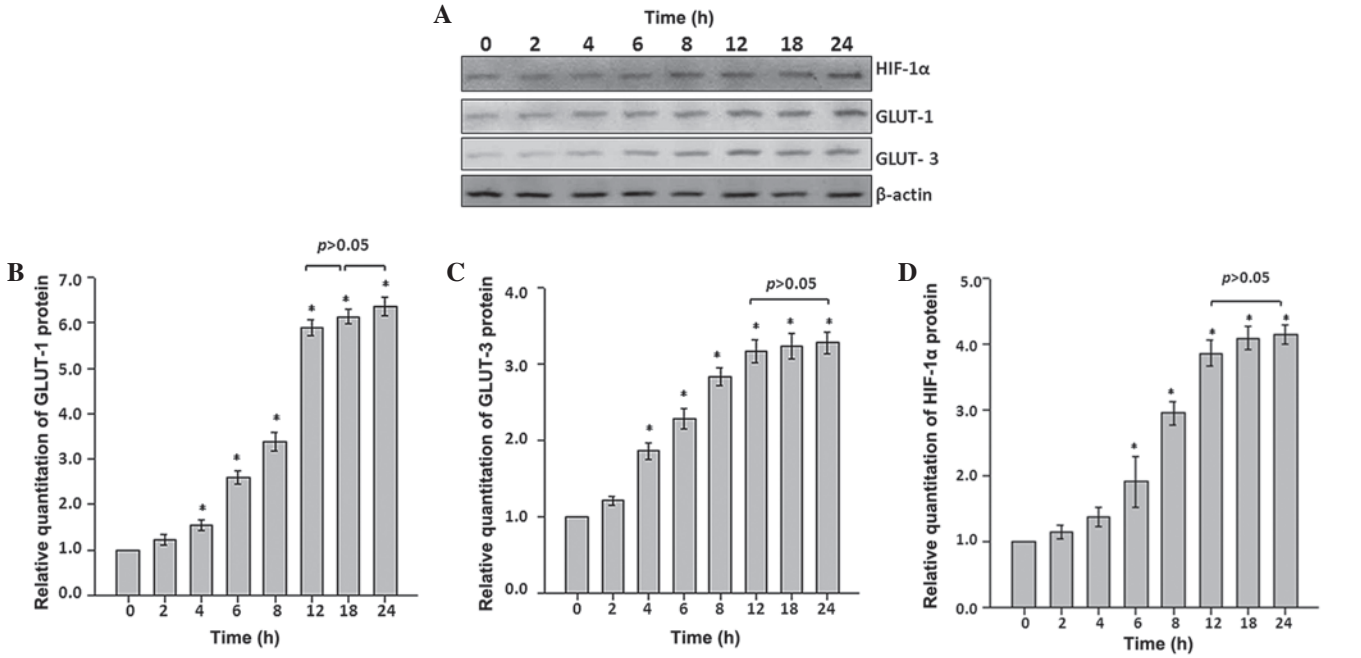

Figure 2. Effects of $CoCl_2$ treatment on protein levels of GLUT-1, GLUT-3 and HIF-1α. Astrocytes were exposed to 100 μM $CoCl_2$ for the presented time courses, the cell lysate was collected and protein expression levels of GLUT-1, GLUT-3 and HIF-1α were determined by western blot analysis using β-actin as the loading control. Values were normalized to β-actin and presented as ratios over control (0 h). (A) Protein expression levels of GLUT-1, GLUT-3, HIF-1α and β-actin were determined by western blot analysis. Ratios of (B) GLUT-1/β-actin, (C) GLUT-3/β-actin and (D) HIF-1α/β-actin were determined at the indicated time points. Results are represented as the mean ± standard deviation of three independent experiments (n=3). *P<0.05 vs. control. GLUT-1/3, glucose transporter-1/3; HIF-α, hypoxia-inducible factor-1α.

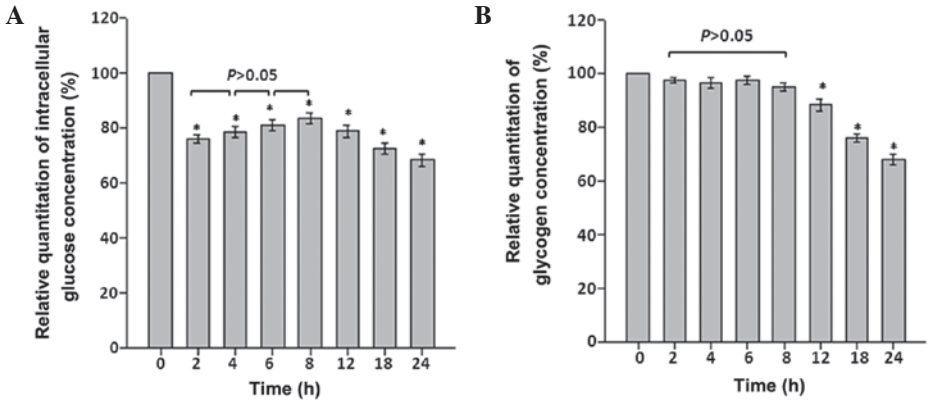

Figure 3. Effects of $CoCl_2$ treatment on glucose uptake activity and glycogen storage. (A) Intracellular glucose concentration was significantly reduced at the 2 h time point following astrocyte exposure to 100 μM $CoCl_2$. (B) Intracellular glycogen concentration was reduced significantly from the 12 h time point onwards. Values are presented as the percentage vs. control (0 h time point), which was expressed as 100%. Data are presented as the mean ± standard deviation of three independent experiments and samples were analyzed in triplicate (n=9). *P<0.05 vs. control.

manner following $CoCl_2$ administration. Expression levels of GLUT-1 and GLUT-3 mRNA increased immediately and significantly in each time interval during the first 12 h time period. Throughout the rest of the recording period, the expression levels for GLUT-1 and GLUT-3 mRNAs remained at ~4.5- and 3.5-fold higher than that of the control (P<0.05 vs. control; Fig. 1A and B).

HIF-1α mRNA expression in astrocytes was also analyzed as an indication of hypoxic stress. HIF-1α mRNA expression exhibited time-dependent alterations similar to those observed with GLUT-1 and GLUT-3. Following exposure to $CoCl_2$ treatment, the astrocytes exhibited a ~2.9-fold increase in HIF-1α mRNA expression during the first 12 h time course, and the increase remained constant throughout the remaining recording period (Fig. 1C).

Effects of $CoCl_2$ treatment on protein levels of GLUT-1, GLUT-3 and HIF-1α. Protein expression levels of GLUT-1, GLUT-3 and HIF-1α were determined by western blot analysis (Fig. 2A). Quantitative analysis demonstrated that treatment with$CoCl_2$ in the first 2 h resulted in no significant differences in GLUT-1 and GLUT-3 protein expression levels in astrocytes (P>0.05 vs. control; Fig. 2B and C); however, a significant increase in the protein expression of GLUT-1 and GLUT-3 occurred from the 4 h time point throughout the experiments (P<0.05 vs. control; Fig. 2B and C). In particular, 6.3- and

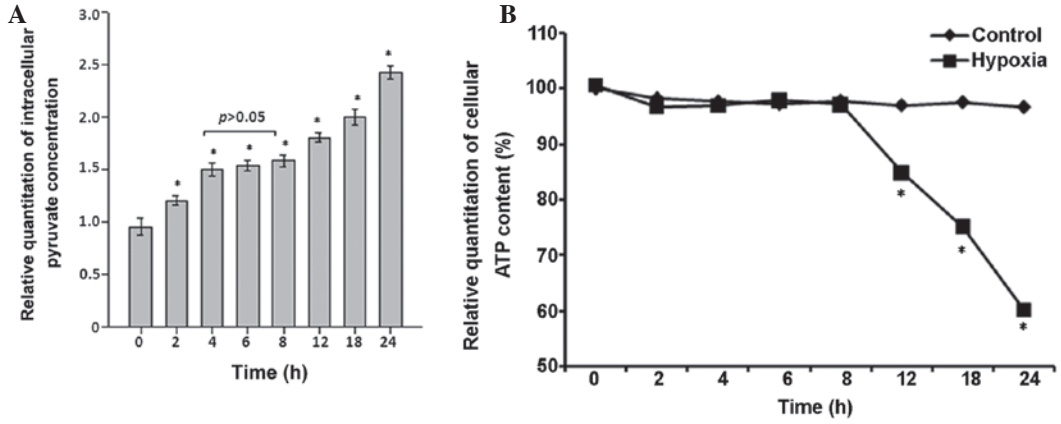

Figure 4. Effects of $CoCl_2$ treatment on intracellular pyruvate concentration and ATP content. (A) Intracellular pyruvate concentration was measured by a colorimetric assay at different time points after astrocytes were exposed to 100 μM $CoCl_2$. Values are presented as the percentage vs. control (0 h time point), which was expressed as 100%. (B) Cellular ATP content was detected by bioluminescence assay subsequent to exposure of astrocytes to 100 μM $CoCl_2$ or serum free medium for the indicated time periods up to 24 h. Results are presented as the mean ± standard deviation of three independent experiments and samples were analyzed in triplicate (n=9). Statistical analyses for cellular ATP content at all time-points between the two groups was performed using an independent-samples t-test. *P<0.05 vs. control. ATP, adenosine triphosphate.

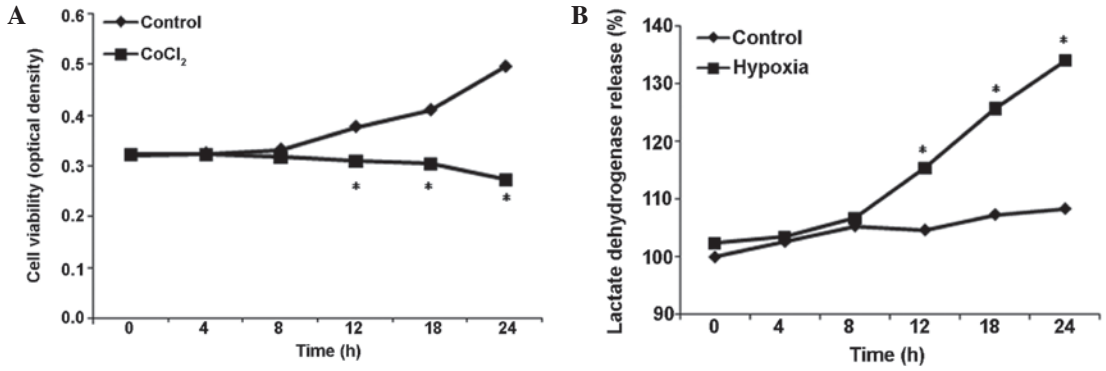

Figure 5. Effects of $CoCl_2$ treatment on cytotoxicity in astrocytes. (A) Cell viability was detected by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay following $CoCl_2$ treatment or serum free medium for the indicated time periods. Values are presented as optical density vs. the control. (B) Lactate dehydrogenase release activity was determined using a colorimetric assay at different time points following treatment with $CoCl_2$ or serum free medium. Values are presented as the percentage vs. 0 h time point of the control, which is expressed as 100%. Data are presented as the mean ± standard deviation of three independent experiments and samples were analyzed in triplicate (n=9). Statistical analyses were conducted at all time-points between the two groups using an independent-samples t-test. *P<0.05 vs. control.

3.2-fold increases for GLUT-1 and GLUT-3, respectively, were observed from the 12 h time point throughout the remaining recording period (Fig. 2B and C).

Additionally, to investigate whether $CoCl_2$ treatment induced GLUT-1 and GLUT-3 activity, and whether HIF-1α behaved in an independent manner, protein expression levels for HIF-1α were determined. As a reflection of hypoxic stress, HIF-1α protein expression behaved similarly to that of GLUT-1 and GLUT-3. There was no statistically significant alteration within the initial 4 h, however a significant increase was identified at the 6 h time point. The increase reached ~4.0-fold at the 12 h time point and remained at that level throughout remaining recording period (P<0.05 vs. control; Fig. 2D).

Effects of $CoCl_2$ treatment on intracellular glucose concentrations and glycogen storage. To determine the effects of $CoCl_2$ treatment on intracellular glucose concentrations and glycogen storage at different time points, intracellular glucose and glycogen concentrations were determined. When astrocytes were exposed to $CoCl_2$ treatment, intracellular glucose concentration immediately fell by >25% between 0 and 2 h (P<0.05). Although an increase occurred between the 4-8 h time points, intracellular glucose and glycogen concentrations were significantly reduced compared with the control by the 24 h time point (P<0.05; Fig. 3A and B).

Effects of $CoCl_2$ treatment on intracellular pyruvate concentration and ATP content. Intracellular pyruvate in astrocytes (Fig. 4A) immediately increased by ~1.2-fold within the first 2 h (P<0.05 vs. control), but remained steady at a level of ~1.6-fold increase relative to the control between 4 and 8 h (P<0.05 vs. control). Subsequently, the intracellular pyruvate concentration further increased from the 12 h time point to ~2.5-fold at the 24 h time point (P<0.05; Fig. 4A).

By contrast, there were no statistically significant differences between the two groups in terms of intracellular ATP content in the first 8 h subsequent to exposure of astrocytes to 100 μM of $CoCl_2$ (P>0.05; Fig. 4B); however, intracellular ATP content significantly reduced in the hypoxia group each subsequent time interval (Fig. 4B).

Effects of $CoCl_2$ treatment on cell viability and cytotoxicity in astrocytes. To further investigate the effects of $CoCl_2$ treatment on cell viability and cytotoxicity in astrocytes, cell viability and LDH release activity were determined. Compared with the control group, cell viability and LDH release activity in the hypoxia group remained at the baseline level for the first 8 h of $CoCl_2$ treatment ($P>0.05$ vs. control; Fig. 5A and B); however, cell viability reduced significantly following exposure to 100 μM $CoCl_2$ for >12 h, while LDH release activity continued to increase in each subsequent time interval.

Discussion

To investigate whether astrocytic expression of GLUT-1 and GLUT-3 is crucial for regulation of glucose uptake under $CoCl_2$ treatment, mRNA and protein expression levels of GLUT-1 and GLUT-3 were determined along with HIF-1α, an indicator of hypoxic stress and a key regulator of hypoxia (9). It has been reported that levels of GLUT-1 and GLUT-3 are controlled at the transcriptional level by HIF-1 (13,26,27). However, under hypoxic conditions, the flux of glucose through the glycolytic pathway may be increased by HIF-1α in order to maintain the requisite ATP levels necessary to sustain life (12). The current study determined that protein and mRNA levels of GLUT-1 and GLUT-3 were elevated in a time-dependent manner under $CoCl_2$ treatment, followed by HIF-1α accumulation resulting from the increased expression. Expression levels of GLUT-1, GLUT-3 and HIF-1α mRNA increased immediately and significantly at each time interval during the first 12 h and remained at the higher levels. Additionally, it was determined that the mRNA expression levels of GLUT-1, GLUT-3 and HIF-1α were higher following treatment with $CoCl_2$, and the regulation occurred at the transcriptional level prior to protein alteration. Under physiological conditions (0 h time point), protein expression of GLUT-1 and GLUT-3 were detected at low levels, and in the first 2 h of $CoCl_2$ treatment, protein expression levels of GLUT-1 and GLUT-3 did not change. However, protein expression levels of GLUT-1 and GLUT-3 increased significantly during the 4-12 h time points and remained at higher levels throughout the remaining recording period ($P<0.05$ vs. control). By contrast, protein expression of HIF-1α remained at baseline levels in the first 4 h of $CoCl_2$ treatment, but significantly increased from the 6-12 h time points and increased further to ~4-fold relative to the control. Therefore, it is possible that GLUT-1 and GLUT-3 may be regulated by HIF-1α; however, they have also been reported to be affected by other regulators, such as connexin 43, c-Src and the Akt/protein kinase A signaling pathway (10,28,29). As hypoxia has been indicated to have significant effects on pH homeostasis due to lactate production and upregulation of glycolysis to maintain ATP production. Na^+/H^+ exchanger isoform 1 regulation and pattern of intracellular pH changes were investigated (data not shown). It had been observed that during the early period (in the first 2 h of $CoCl_2$ treatment), both NHE1 activity and pHi dropped immediately with reducing NHE1 mRNA expression, whereas expression levels of NHE1 protein were not altered. In the later period of $CoCl_2$ treatment, activity of NHE1 and value of pHi markedly increased, and was associated with increased mRNA and protein expression levels of NHE1 (data not shown).

GLUTs are critical for cell survival under hypoxic conditions. It is controversial whether GLUT-3 is expressed in astrocytes (16,18). The present study indicates that GLUT-3 was detected at extremely low levels in cultured astrocytes under physiological conditions, however whether GLUT-3 may be induced in adult brain *in vivo* still remains unclear. GLUT-3 transports extracellular glucose ~7 times faster than GLUT-1 (19), as GLUT-3 only takes up extracellular glucose and does not release intracellular glucose to the extracellular space, even if the intracellular glucose concentration is higher than the extracellular concentration (8). Additionally, compared with GLUT-3 mRNA and protein levels, $CoCl_2$ treatment induced a higher increase in GLUT-1 mRNA and protein levels ($P<0.05$). It is likely that GLUT-1 is important for the increased glucose influx into astrocytes (30); therefore, these results indicate that inducing GLUT-3 may in turn support GLUT-1 activities, and promote the enhancement of glucose uptake and transport into the astrocytes to meet cellular energy requirements for maintaining cell viability during hypoxia (10,31).

By contrast, glucose was quickly consumed to recover or sustain the homeostasis of cells; therefore, intracellular glucose and glycogen levels were then investigated. Intracellular glucose concentration fell immediately by ~25% and increased only marginally between the 2-8 h time points. Intracellular glucose concentration then continued to reduce throughout the remaining recording period. Similarly, intracellular glycogen was reduced in the first 8 h and then significantly reduced in the remaining hours, although protein expression levels of GLUT-1 and GLUT-3 were increased. To explain this phenomenon, increased glycolysis, which serves to ameliorate metabolic disorders following hypoxia, may be responsible for breaking down glucose into pyruvate as one of the means by which ATP is generated (32).

To assess this hypothesis, the time-dependent manner by which glucose was consumed following astrocyte exposure to 100 μM $CoCl_2$ was determined for distinct time periods. It was determined that the intracellular pyruvate of astrocytes increased immediately within the first 2 h following $CoCl_2$ treatment, and then remained at a steady level of ~1.6-fold relative to the control during the 4-8 h time points, and continued to significantly increase throughout the remaining recording period, reaching ~2.5-fold at the 24-h time point relative to the control. By contrast, intracellular ATP content did not change within the first 8 h but began to significantly reduce during the remaining hours. It has been reported that $CoCl_2$ exposure and oxygen deprivation lead to ATP depletion in astrocyte cultures; however, ATP is reduced to a lower extent by hypoxia, suggesting that $CoCl_2$ has additional cellular targets (26). Furthermore, $CoCl_2$ is an established blocker of voltage-gated calcium channels, which may explain its inhibition of morphological differentiation induced by cyclic adenosine monophosphate in astrocytes (26,33). The observations of the present study suggest that, under $CoCl_2$ treatment, increased glucose uptake allows astrocytes to utilize more glucose for increased glycolysis, which may also be increased by HIF-1α to maintain the requisite ATP levels essential for cell survival and necessary to sustain life, resulting in an increase in pyruvate and lactate production.

Glycolysis generates a net gain of only two molecules of ATP per glucose molecule, a markedly smaller quantity of energy compared with the net gain of 38 molecules of ATP that is produced by respiration. Thus, normal cells gain only 10% of their energy through glycolysis (11,34) and require more glucose uptake to obtain sufficient energy. During the first 8 h of $CoCl_2$ treatment, increased glucose uptake compensated for glucose consumption in the cells by maximizing their energy production. Thus, there was no significant change in cell viability and LDH release compared with minor changes in intracellular glycogen storage and ATP content; however, due to the increase in glycolysis, the intracellular pyruvate concentration increased and the glucose concentration was reduced. Intracellular glucose concentration recovered only marginally during the 4-8 h time points as a result of increased expression levels of GLUT-1 and GLUT-3; however, it remained at a lower level compared with the control. However, after 8 h, the increase in glucose uptake did not meet the demand of enhanced glycolysis, leading to a breakdown of glucose homeostasis, depletion of glycogen, deficit of ATP and the accumulation of pyruvate. As a regular supply of energy is essential to maintain normal cell function, the loss of the energy supply, even for a short period of time, results in cell death (7). In the current study, reduced cell viability and increased LDH release activity were observed 8 h subsequent to $CoCl_2$ treatment.

In conclusion, the current study indicated that glucose homeostasis in astrocytes under $CoCl_2$ treatment is time-dependent. $CoCl_2$ treatment exerted early effects (in the first 8 h) on glucose homeostasis, leading to minor alterations in LDH release activity, intracellular glycogen storage and ATP content; however a significant increase in pyruvate concentration and a reduction in glucose concentration in astrocytes was observed. This may be a mechanism for the maintenance of cell viability as a result of increased glycolysis. However, 8 h of treatment led to increased LDH release activity, accumulating pyruvate concentration and reduced intracellular glucose concentration, a shortage of ATP content, and a deficit in glycogen storage associated with reduced cell viability. It was also determined that the expression levels of GLUT-1 and GLUT-3 were regulated by $CoCl_2$ treatment in a time-dependent manner, and were associated with the regulation of HIF-1α. Therefore, the present study provides novel evidence that the regulation of GLUT-1 and GLUT-3 may be responsible for alterations in cell viability and energy production under $CoCl_2$ treatment, as a mimic of hypoxia in astrocytes, and these observations emphasize the relevance of hypoxia in GLUT function regulation and glucose homeostasis in astrocytes. Further investigation is required to elucidate the mechanisms by which injury occurs following human stroke, to develop neuroprotective strategies and to determine an optimal time frame to commence therapeutic procedures following a stroke.

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References

1. Cunningham LA, Candelario K and Li L: Roles for HIF-1α in neural stem cell function and the regenerative response to stroke. Behav Brain Res 227: 410-417, 2012.
2. Wang C, Wang Z, Zhang X, Zhang X, Dong L, Xing Y, Li Y, Liu Z, Chen L, Qiao H, *et al:* Protection by silibinin against experimental ischemic stroke: up-regulated pAkt, pmTOR, HIF-1α and Bcl-2, down-regulated Bax, NF-κB expression. Neurosci Lett 529: 45-50, 2012.
3. Zemke D, Smith JL, Reeves MJ and Majid A: Ischemia and ischemic tolerance in the brain: An overview. Neurotoxicology 25: 895-904, 2004.
4. Ratan RR, Siddiq A, Smirnova N, Karpisheva K, Haskew-Layton R, McConoughey S, Langley B, Estevez A, Huerta PT, Volpe B, *et al*: Harnessing hypoxic adaptation to prevent, treat, and repair stroke. J Mol Med Berl 85: 1331-1338, 2007.
5. Vemula S, Roder KE, Yang T, Bhat GJ, Thekkumkara TJ and Abbruscato TJ: A functional role for sodium-dependent glucose transport across the blood-brain barrier during oxygen glucose deprivation. J Pharmacol Exp Ther 328: 487-495, 2009.
6. Agrawal M, Kumar V, Kashyap MP, Khanna VK, Randhawa GS and Pant AB: Ischemic insult induced apoptotic changes in PC12 cells: protection by trans resveratrol. Eur J Pharmacol 666: 5-11, 2011.
7. Reagan LP, Magariños AM, Lucas LR, van Bueren A, McCall AL and McEwen BS: Regulation of GLUT-3 glucose transporter in the hippocampus of diabetic rats subjected to stress. Am J Physiol 276: E879-E886, 1999.
8. Iwabuchi S and Kawahara K: Inducible astrocytic glucose transporter-3 contributes to the enhanced storage of intracellular glycogen during reperfusion after ischemia. Neurochem Int 59: 319-325, 2011.
9. Badawi Y, Ramamoorthy P and Shi H: Hypoxia-inducible factor 1 protects hypoxic astrocytes against glutamate toxicity. ASN Neuro 4: 231-241, 2012.
10. Valle-Casuso JC, González-Sánchez A, Medina JM and Tabernero A: HIF-1 and c-Src mediate increased glucose uptake induced by endothelin-1 and connexin43 in astrocytes. PLoS One 7: e32448, 2012.
11. Pereira KM, Chaves FN, Viana TS, Carvalho FS, Costa FW, Alves AP and Sousa FB: Oxygen metabolism in oral cancer: HIF and GLUT-s. Oncol Lett 6: 311-316, 2013 (Review).
12. Seagroves TN, Ryan HE, Lu H, Wouters BG, Knapp M, Thibault P, Laderoute K amd Johnson RS: Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. Mol Cell Biol 21: 3436-3444, 2001.
13. Kinni H, Guo M, Ding JY, Konakondla S, Dornbos D III, Tran R, Guthikonda M and Ding Y: Cerebral metabolism after forced or voluntary physical exercise. Brain Res 1388: 48-55, 2011.
14. Brix B, Mesters JR, Pellerin L and Jöhren O: Endothelial cell-derived nitric oxide enhances aerobic glycolysis in astrocytes via HIF-1α-mediated target gene activation. J Neurosci 32: 9727-9735, 2012.
15. Rouach N, Koulakoff A, Abudara V, Willecke K and Giaume C: Astroglial metabolic networks sustain hippocampal synaptic transmission. Science 322: 1551-1555, 2008.
16. Duelli R and Kuschinsky W: Brain glucose transporters: Relationship to local energy demand. News Physiol Sci 16: 71-76, 2001
17. Nijland PG, Michailidou I, Witte ME, Mizee MR, van der Pol SM, van Het Hof B, Reijerkerk A, Pellerin L, van der Valk P, de Vries HE and van Horssen J: Cellular distribution of glucose and monocarboxylate transporters in human brain white matter and multiple sclerosis lesions. Glia 62: 1125-1141, 2014.

18. Cidad P, Garcia-Nogales P, Almeida A and Bolaños JP: Expression of glucose transporter GLUT-3 by endotoxin in cultured rat astrocytes: the role of nitric oxide. J Neurochem 79: 17-24, 2001.
19. Maher F, Davies-Hill TM and Simpson IA: Substrate specificity and kinetic parameters of GLUT-3 in rat cerebellar granule neurons. Biochem J 315: 827-831, 1996.
20. Efrati S, Fishlev G, Bechor Y, Volkov O, Bergan J, Kliakhandler K, Kamiager I, Gal N, Friedman M, Ben-Jacob E and Golan H: Hyperbaric oxygen induces late neuroplasticity in post stroke patients - randomized, prospective trial. PLoS One 8: e53716, 2013.
21. Saxena S, Shukla D and Bansal A: Augmentation of aerobic respiration and mitochondrial biogenesis in skeletal muscle by hypoxia preconditioning with cobalt chloride. Toxicol Appl Pharmacol 264: 324-334, 2012.
22. Björklund O, Shang M, Tonazzini I, Daré E and Fredholm BB: Adenosine A1 and A3 receptors protect astrocytes from hypoxic damage. Eur J Pharmacol 596: 6-13, 2008.
23. McCarthy KD and de Vellis J: Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol 85: 890-902, 1980.
24. Karovic O, Tonazzini I, Rebola N, Edström E, Lövdahl C, Fredholm BB and Daré E: Toxic effects of cobalt in primary cultures of mouse Astrocytes Similarities with hypoxia and role of HIF-1a. Biochem Pharmacol 73: 694-708, 2007
25. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
26. Schubert D: Glucose metabolism and Alzheimer's disease. Ageing Res Rev 4: 240-257, 2005.
27. Jones NM and Bergeron M: Hypoxic preconditioning induces changes in HIF-1 target genes in neonatal rat brain. J Cereb Blood Flow Metab 21: 1105-1114, 2001.
28. Wang KR, Jiang T, Wu TT, Zhou SH, Yao HT, Wang QY and Lu ZJ: Expression of hypoxia-related markers in inflammatory myofibroblastic tumors of the head and neck. World J Surg Oncol 11: 294, 2013.
29. Papa Pde C, Sousa LM, Silva Rdos S, de Fátima LA, da Fonseca VU, do Amaral VC, Hoffmann B, Alves-Wagner AB, Machado UF and Kowalewski MP: Glucose transporter 1 expression accompanies hypoxia sensing in the cyclic canine corpus luteum. Reproduction 147: 81-89, 2014.
30. Yamada T, Uchida M, Kwang-Lee K, Kitamura N, Yoshimura T, Sasabe E and Yamamoto T: Correlation of metabolism/hypoxia markers and fluorodeoxyglucose uptake in oral squamous cell carcinomas. Oral Surg Oral Med Oral Pathol Oral Radiol 113: 464-471, 2012.
31. Xu O, Li X, Qu Y, Liu S, An J, Wang M, Sun Q, Zhang W, Lu X, Pi L, *et al:* Regulation of glucose transporter protein-1 and vascular endothelial growth factor by hypoxia inducible factor 1α under hypoxic conditions in Hep-2 human cells. Mol Med Rep 6: 1418-1422, 2012.
32. Dornbos D III, Zwagerman N, Guo M, Ding JY, Peng C, Esmail F, Sikharam C, Geng X, Guthikonda M and Ding Y: Preischemic exercise reduces brain damage by ameliorating metabolic disorder in ischemia/reperfusion injury. J Neurosci Res 91: 818-827, 2013.
33. MacVicar BA: Morphological differentiation of cultured astrocytes is blocked by cadmium or cobalt. Brain Res 420: 175-177, 1987.
34. Eckert AW, Lautner MH, Schütze A, Taubert H, Schubert J and Bilkenroth U: Coexpression of hypoxia-inducible factor-1α and glucose transporter-1 is associated with poor prognosis in oral squamous cell carcinoma patients. Histopathology 58: 1136-1147, 2011.