Cerebral hypoxia is known to be a risk factor for neurodegenerative diseases, and it implicates many pathological disorders in brain including neuron energy depletion, excitotoxicity injury and a great number of free radicals production. An understanding of the mechanism of neuronal damage due to hypoxia in the neuronal cells is necessary to clarify the mechanism of neuronal damage. The therapeutic efforts that aim to attenuate brain disruptions caused by hypoxia may be helpful in the treatment of neurodegenerative diseases.
It is well known that cyanide is a hypoxic agent, which can inhibit activities of many enzymes such as cytochrome oxidase, peroxidase, succinic dehydrogenase, etc., especially those sensitive to the cytochrome oxidase. Cyanide can combine with Fe$^{3+}$ of cytochrome oxidase and block Fe$^{3+}$ reduction into Fe$^{2+}$ and inhibit electron transmitting of respiratory chain, and then suppress the energy production of mitochondria. The brain is critically dependent on a continuous energy supply generated through oxidative phosphorylation in respiratory chain and is vulnerable to damage by hypoxia. Both in vivo and in vitro studies have demonstrated that the brain is very sensitive to cyanide and led to alterations in neurotransmitter stores, and generation of reactive oxygen, which are the potential mediators of cyanide neurotoxicity. The effects induced by cyanide are analogous to some metabolite disorders seen in patients who suffer from cerebral hypoxia. Thus cyanide can be used as a hypoxic agent to develop an animal model to mimic the state hypoxic neurodegenerative diseases in human.

Clonal cell lines, the rat pheochromocytoma line PC12 cells, provide useful model for the investigation of neuronal injury. The clonal PC12 cells contain many types of nerve cells as well as glial populations, and afford homogeneous populations and the ease of manipulation and degree of control that may be exerted over the extracellular milieu providing definite advantages over animal/primary neuronal culture experiments.

Scutellaria baicalensis Georgi is a common traditional Chinese medicine, which has efficacy in heat clearing and damp drying, and purging fire to eliminate toxin. Its extracts possess defervescence, anti-inflammatory, anti-virus activities and enhance immunity. SSF, a group of flavonoid compounds isolated from dried aerial part of Scutellaria baicalensis Georgi, has been proven to be a promising agent for palliative treatment of neurodegenerative diseases. In previous studies, SSF has shown improvements in brain hypoxia, memory impairment and neuronal damage in experimental animal models and protection of oxidative injury on PC12 cells in vitro. We carried out this study to examine the effects of SSF on PC12 cells survival, energy metabolites and oxidative-antioxidant system induced by hypoxia with direct application of KCN.

**Material & Methods**

**Materials:** The purity of SSF was about 61.88 per cent. In order to eliminate the cytotoxicity by some non-specific factors such as pH, osmotic pressure, tannin and inorganic salt in the drug, we utilized the rat serum with drug to investigate the protection of SSF on PC12 cells damage induced by KCN. The value of SSF in rat serum was 465.88 µg/ml serum assayed by HLPC and the preparation of serum with drug was as previous description.

SSF was prepared by the Department of Phytochemistry, Institute of Traditional Chinese Medicine, Chengde Medical College, as described previously. The RPMI 1640 medium and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma, USA. The foetal bovine serum and horse serum were provided by the Institute of Tianjin Blood (China). The reagent kits for measurement of malondialdehyde (MDA), superoxide dismutase (SOD), Na$^+$-K$^+$-ATPase and lactate dehydrogenase (LDH) were purchased from Nanjing Jiancheng Institute of Biological Engineering and Zhongsheng Beikong Bio-technology and Science Inc. (China). The culture 24- and 96-well plastic plates were obtained from Gibco, (America).

**Cell culture:** PC12 cells were a gift from Professor Chen Shiming from Institute of Materia Medica of Chinese Academy of Medical Science and were maintained at 37°C in a humidified atmosphere containing 5 per cent CO$_2$. Cells (1 ml) were seeded into phenol red-free RPMI 1640 medium, supplemented with 5 per cent heat-inactivated bovine serum, 5 per cent heat-inactivated horse serum, 100 U/ml penicillin and 100 µg/ml streptomycin and maintained at 37°C in a humidified atmosphere containing 5 per cent CO$_2$. The experiments were carried out 48 h after cells were seeded. When assaying the cell survival and LDH release, the cells were seeded into poly-L-lysine-coated 96-well plastic plates with 0.15 ml at density of $10^4$ cells per ml.

**KCN and drug treatment:** KCN was stored at 4°C; 10 mM stock solutions were prepared in phosphate-buffered saline (PBS) on the day of application to the cultures. The 10 mM KCN was further diluted to 5 mM for addition to the cultures. The PC12 cells were pre-incubated with serum with SSF (18.98, 37.36 and 75.9 mg/ml) 24 h before the exposure to KCN. Assays for cell survival, LDH release, free radicals and Na$^+$-K$^+$-ATPase activity were performed 30 min after the cultures were exposed to 200 µM KCN. Each independent experiment was carried out in five or six wells. The dose of drug selected was according to our previous paradigm. The control cells were treated with...
Measurement of Na⁺-K⁺-ATPase MDA and SOD: For assay of Na⁺-K⁺-ATPase and MDA and SOD, the cultures were washed with ice-cold PBS and then pooled in 0.1 M PBS-0.05 mM EDTA-buffered solution. The cell suspension was frozen at -85 °C for 2 h and rapidly melted at 37 °C. The same process was performed 3 times. The cell suspension was centrifuged for 1 h at 10000 g at 4°C. Na⁺-K⁺-ATPase activity in supernatant was determined by measuring the rate of formation of phosphoric acid from ATP. The process of measurement was according to the instructions of the manufacturer. The Na⁺-K⁺-ATPase activity was calculated as follows:

\[ \text{Na⁺-K⁺-ATPase activity in cell supernatant} = \left( \frac{A_j - A_i}{A_j} \right) \times C \times D \times 6 \times T \left( \mu \text{mol} \ p/\text{ml/h} \right). \]

Where \( A_i \) and \( A_j \) are the absorbance of sample and standard, respectively. \( D \) is the dilute times of sample and \( T \) is the protein concentration of the sample.

MDA content assay: The content of MDA was determined by using the thiobarbituric acid method. Two volumes of thiobarbituric acid reagent were added to the sample supernatant and boiled for 40 min at 100°C, and after cooling and centrifugation at 3000 × g for 10 min, the absorbance of each supernatant was measured at 532 nm. The level of MDA was calculated as follows:

\[ MDA \text{ level} = \left( \frac{A_{j} - A_{i}}{A_{j} - A_{i}} \right) \times C \left( \mu \text{mol/l} \right). \]

Where \( A_i \) and \( A_j \) are the absorbance of sample and standard blank; \( A_{i} \) and \( A_{j} \) are the absorbance of standard and standard blank, respectively. \( C \) is the standard concentration of MDA.

SOD activity assay: SOD activity was measured by xanthine-xanthine oxidase method. The supernatant was added to xanthine-xanthine oxidase reagent and incubated for 40 min at 37°C. SOD was added to stop the reaction, followed by measurement of absorbance at 550 nm. One unit of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50 per cent. The SOD activity was calculated as follows:

\[ \text{SOD activity} = \left( \frac{A_{j} - A_{i}}{A_{j} - A_{i}} \right) \times 50 \% \times D \left( \text{kU/ml} \right). \]

Where \( A_i \) and \( A_j \) are the absorbance of control and sample, respectively. \( D \) is the dilute times of sample in the reactive system.

Results & Discussion

PC12 cells showed a typical swelling and significant decrease in cell number, disappearance in cells reticular formation and most cells lost their neurites and assumed a rounded shape after exposure to 200 µM KCN for 30 min (Fig. 1 A, B). In contrast, the cultured PC12 cells received the same treatment of KCN in the presence of SSF (18.98, 37.36 and 75.92 µg/ml) appeared dramatically preserved, and the effect of SSF appeared to be dose-dependent (Fig. 1C-1E).

There is evidence showing that MTT is the earliest identified marker for the cytotoxic effects. Active mitochondria of living cells can cleave MTT to produce formazan, the amount of which directly reflects the living cell number. In the present study, PC12 cells survival as determined by MTT reduction, was markedly decreased after exposure to 200 µM KCN for 30 min. The decrease in MTT reduction of injured cells by KCN was notably reversed by serum with SSF at dose of 18.98, 37.36 and 75.92 µg/ml and the effect was dose-dependent (Fig. 2). Both morphological observation and MTT assay have shown protection by SSF against KCN insult in cultured PC12 cells.

The present results show that the cytotoxicity of cultured PC12 cells induced by KCN was accompanied by alteration in free radicals and Na⁺-K⁺-ATPase activity. The cells exposed 200 µM KCN for 30 min...
produced reductions in the activities of SOD and Na\(^+\)-K\(^+\)-ATPase by 47.2 and 58.33 per cent, \((P<0.05)\). Meanwhile, the content of intracellular MDA and LDH release in PC12 with KCN treatment were 4.48 and 1.66-folds higher than those of control cells. The deteriorative changes in MDA content and LDH release and SOD and Na\(^+\)-K\(^+\)-ATPase activities in KCN treated PC12 cells were significantly attenuated by pre-treatment with serum with SSF at the dose of 18.98, 37.36 and 75.92 µg/ml for 24 h (Table).

Many studies demonstrated that hypoxia primarily contributes to the disorders in the physiology, biochemistry and morphology of brain\(^{18,19}\). The neuronal damage after cyanide exposure is attributed primarily...
to its hypoxic action, reductions in cellular metabolism and energy through inhibition of cytochrome oxidase. The cytochrome oxidase is an important enzyme of oxidative phosphorylation in respiratory chain. The brain needs continuous supply of energy, and the structure and function of brain will suffer when there is a deficit in energy supply20,21. In the present study, KCN caused significant cytotoxicity in PC12 cells line. Following 30 min PC12 cells exposure to KCN, the morphology of the cell showed noted pathological changes including a significant decrease in cell number; disappearance in cells reticular formation and the loss in neurites of most cells. The cell survival (MTT reduction) and MTT-dependent LDH release were also affected. LDH is an intracellular enzyme, the amount of which released into the cultured medium indicates the state of cell injury13,17. Our data suggest that PC12 cells were extremely sensitive to KCN induced cell injury. However, pre-treatment with the rat serum with SSF significantly attenuated KCN-induced cells toxicity and gave a noticeable morphological protection. The reduction in the cell survival and the increase in LDH release were also reversed by SSF in a dose dependent manner. The effect of SSF on cell survival and LDH release ran parallel with the effect on cell pathological attenuation.

There is confluence of opinion that cellular events involving free radical oxidative stress may be one basic pathway leading to cell degeneration. A series of investigations suggested that increased oxidative stress and disturbed defensive mechanisms occur in the brain in neurodegenerative diseases4. The cerebral ischaemia/hypoxia inevitably results in metabolic disruptions of free radical in central nervous system. The brain is liable to be peroxidated by free radicals as a result of brain’s high oxygen consumption rate, abundant lipid content and the related paucity of anti-oxidative enzymes4,22. Cyanide inhibits cytochrome oxidase of respiratory chain to block the effective ATP production and advances to impact mitochondrial function. Free radical (O2 ), formed as a byproduct of mitochondrial respiration, leads to damage or destruction of a variety of tissues. Consequences of excessive reactive oxygen species are oxidative damages to lipid, protein and DNA. The level of MDA is generally considered to reflect the cell injury. In addition, antioxidants such as SOD not only prevents the accumulation of cellular free radicals but also reduces the cytotoxicity4,23.

It is well known that the Na+-K+-ATPase consumes 50 per cent of the energy supply in the CNS and reduction of Na+-K+-ATPase activity is characterized by the onset of cerebral ischaemia due to intracellular ATP depletion24,25. Thus, the Na+-K+-ATPase activity is sensitive to the ATP supply. It is reported that hypoxia can result in free radicals increase in mitochondria and a decrease in Na+-K+-ATPase activity, which is capable of

| Table. Effects of SSF on LDH release, MDA content, Na+-K+-ATPase and SOD activities in PC12 cells |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Group           | LDH release     | Na+-K+-ATPase   | SOD       | MDA             |
|                 | (U/l)           | (µmol p/ml/h)   | (U/ml)   | (nmol/ml)       |
| Control group   | 54.52±2.43      | 73.60±2.23      | 16.25±0.72| 0.67±0.07       |
| KCN group       | 90.28±12.06*    | 30.67±4.07*     | 8.67±1.67*| 3.00±0.76*      |
| SSF 18.98 µg/ml | 80.46±9.97      | 37.75±5.28*     | 11.07±0.83*| 2.14±0.26*      |
| μg/ml 37.96     | 69.43±6.23**    | 39.49±3.29**    | 11.63±0.65**| 1.71±0.28**     |
| 75.92           | 62.54±8.74**    | 63.94±5.46**    | 14.10±0.57**| 0.76±0.28**     |

Data were expressed as means±SE, (n = 5-6). *P<0.05, **P<0.01 compared with control group; †P<0.05, ‡P<0.01, compared with KCN group.
mediating cell degeneration and death, and is possibly involved in related diseases\(^{25,26}\). Thus, the change of Na\(^+\)-K\(^+\)-ATPase activity can partly indicate the correlation between hypoxia and cells toxicity. The SSF exerts neuronal protections due to its polyphenol structure, and the capacity of flavonoids to suppress the activity of several enzymes exerting a net indirect antioxidant effect in addition to the direct scavenging of free radicals\(^{27}\).

In conclusion, our findings demonstrate that SSF might be beneficial for ameliorating KCN-induced cytotoxic injury in the PC12 cells. The action of SSF against hypoxia-induced disturbances may have potential in the treatment of neurodegenerative diseases.

**Acknowledgment**

Authors acknowledge the State Administration of Traditional Chinese Medicine, People’s Republic of China (No. 02-03-ZP18) and Hebei Provincial Education Department of China (No. 20015) for financial support.

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