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Protection of hippocampal slices against hypoxia/hypoglycemia injury by a Gynostemma *pentaphyllum* extract

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Abstract

In transverse hippcampus slices a short period of hypoxia/hypoglycemia induced by perfusion with O_2/glucose-free medium caused early loss and incomplete restoration of evoked field potentials to only 50% in the CA_1 region. We report about a study investigating the effect of an ethanolic *Gynostemma pentaphyllum* extract in this system. When given with reperfusion the extract completely protected the cells of the slices from functional injury. The extract also protected at the subcellular level isolated mitochondria which had been subjected to hypoxia/reoxygenation in combination with elevated extramitochondrial Ca^{2+} concentration from functional injury. In isolated mitochondria the extract protected from Ca^{2+}-induced opening of the mitochondrial permeability transition pore and reduced lipid peroxidation. Our data demonstrate that the ethanolic extract of *Gynostemma pentaphyllum* has a high potential to protect from ischemia/reperfusion injury. It should be beneficial as prophylactic nutrition supplement and during revascularization of arterial blood vessels from stroke and other ischemic events such as coronary occlusion.

Keywords: *Gynostemma pentaphyllum*; Hypoxia/hypoglycemia; Mitochondria; Mitochondrial permeability transition pore

Introduction

In the course of ischemia/reperfusion several cellular processes are initiated that mediate functional injury and cell death in the infarct area. Among them are calcium overload (Murphy and and Steenbergen 2008), oxidative and nitrosive stress (Halestrap et al. 2007), decrease in mitochondrial ATP production (Halestrap et al. 2007), induction of the intrinsic pathway of programmed cell death (Lang and McCullough 2008), and membrane permeabilization (Kroemer et al. 2007). Attempts to target cellular processes during reperfusion by applying antioxidants, inhibitors of calcium transport or inhibitors of apoptosis were only partially beneficial (Perlmann 2006). Moreover, treatments targeting such cellular processes did not reach clinical approval. Consequently, the clinical intervention after ischemic stroke or heart attack is actually restricted to enable reperfusion by administering tissue plasminogen activator which stimulates the degradation of fibrin (Stroke Study Group 1995).

It is very unlikely that one single substance can completely protect against cellular injury because of the complex scenario during ischemia/reperfusion. To improve the outcome of the treatment targeting cellular processes, the combination of different substances is a

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promising strategy. In this context, the application of multi component extracts of special herbs could be an attractive approach.

In fact, complex mixtures derived from herbs had been used in traditional Chinese medicine to treat patients in stroke therapy. More than 100 Chinese medicines had been used for stroke prevention and therapy. The broad spectrum of effects includes antithrombotic and/or thrombolytic activity, improvement of blood circulation, acceleration of blood flow and microcirculation, inhibition of lipid peroxidation, and protection during ischemia/reperfusion. Some of the ingredients and their effects had been characterized. Inhibition of NF-κB signalling, decrease in intracellular Ca^{2+} concentration by ginsenosides due to increased ATPase activity, and inhibition of free-radical generation by ginsenosides had been demonstrated (for review of these effects see Gong and Sucher 2002). Although it is known that *Gynostemma pentaphyllum* also contains ginsenosides, this particular herb had not yet been used in stroke therapy.

Gynostemma pentaphyllum is a wild growing plant that had been used in Asian countries in traditional medicine. Main components of extracts from *Gynostemma pentaphyllum* are as much as 82 different gypenosides, several amino acids and vitamins, and trace elements (Deng et al. 1994). A broad spectrum of beneficial effects had been reported including antioxidative activity (Li et al. 1993; Shang et al. 2006), regulation of blood pressure (Tanner et al. 1999), immune regulatory activity (Hou et al. 1991; Huang et al. 2007a, b), adhesion inhibition (Huang et al. 2007a, b), anti allergic activity (Huang et al. 2008), inhibition of the microsomal Na^+ and K^+-ATPase in brain and heart (Han et al. 2007), anti cancer activity (Han et al. 1995; Chen et al. 2008; Lu et al. 2008; Wang et al. 2002), anti-hyperlipidemic and hypoglycemic activity (Megalli et al. 2006), and regulation of nitric oxide metabolism (Aktan et al. 2003; Tanner et al. 1999).

Here we report about the effect of an ethanolic extract from *Gynostemma pentaphyllum* applied in an ischemia/reperfusion *in vitro* model. Therefore, hippocampal slices were treated with the extract after reversible hypoxia/hypoglycemia and evoked field potentials were measured as functional parameter. Additionally, isolated mitochondria were subjected to hypoxia/reoxygenation in combination with elevated Ca^{2+} concentrations in the presence of the *Gynostemma pentaphyllum* extract and respiration was analysed to document mitochondrial function. Furthermore, the antioxidative potential of the extract against oxidative stress was separately evaluated in a mitochondrial model of iron-ascorbate-induced lipid peroxidation; and the ability of the extract to prevent Ca^{2+}-induced opening of the mitochondrial permeability transition pore was tested by swelling experiments with isolated mitochondria.

Materials and methods

Materials

The standardized *Gynostemma pentaphyllum* extract powder was obtained by extraction of dried aerial parts with 75% ethanol/25% water and was generously received from Herbasin (Shenyang) Co., Ltd. (China). All chemicals were of analytical grade.

TLC- and HPLC- fingerprint-analysis

TLC-fingerprint analysis (Fig. 1 A)

Extraction: 5 mg of the dried ethanolic extract (75% ethanol/25% water) were dissolved in 1 ml of ethanol and filtered over Millipore filtration unit-type 0.45 µm. Reference compound: 0.5 mg of Ginsenoside Rb1 was dissolved in 0.5 ml methanol. Separation parameters: Plate: HPTLC Silica gel 60 F254, (Merck). Applied amount: *Gynostemma pentaphyllum* extract: 20 µl, reference compound: 10 µl. Solvent system: Chloroform/acetic acid/methanol/water 60/32/12/8. Detection: anisaldehyd-sulphuric acid reagent: 0.5 ml anisaldehyd is mixed with 10 ml glacial acetic acid, followed by 85 ml methanol and 5 ml concentrated sulphuric acid, in that order. The TLC-plated is sprayed with about 10 ml, heated at 100 °C for 10 min, then evaluated in VIS. The reagent has only limited stability and is no longer useable when the colour has turned to red-violet.

HPLC-fingerprint analysis (Fig. 1 B)

Sample preparation: 5 mg of the dried ethanolic extract (75% ethanol/25% water) were dissolved in 1 ml of ethanol, filtered over Millipore® filtration unit, type 0.45 µm, and injected into the HPLC apparatus. Injection volume: *Gynostemma pentaphyllum* extract: 20.0 µl. HPLC parameter: Apparatus: MERCK HITACHI D-6000 A Interface, MERCK HITACHI L-4500 A Diode Array Detector, MERCK HITACHI AS-2000 Autosampler, MERCK HITACHI L-6200 A Intelligent Pump. Separation column: LiChroCART® 250-4 LiChrospher® 100 RP-18 (5 µm) (Merck), Precolumn: LiChroCART® 4-4 LiChrospher® 100 RP-18 (5 µm) (Merck). Solvent: A: dist. Water (Millipore Ultra Clear UV plus® filtered), B: acetonitrile (Fa. VWR). Gradient: 5–100% B in 60 min, total runtime: 60 min. Flow: 0.8 ml/min. Detection: 203 nm.

Animals

The experiments were conducted with 8 weeks old male Wistar rats (Tierzucht Schönwalde, Germany). The animals were kept under controlled laboratory conditions (light regime of 12 h light/12 h dark, temperature 20 ± 2 °C, humidity 55–60%). The animals were housed