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A potent inhibition of oxidative stress induced gene expression in neural cells by sustained ferulic acid release from chitosan based hydrogel

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ABSTRACT

Traumatic brain injury (TBI) is an extremely cataclysmic neurological disorder and the inhibition of oxidative stress following TBI could effectively protect the brain from further impairments. An injectable thermosensitive chitosan/gelatin/β-Glycerol phosphate (C/G/GP) hydrogel for the controlled release of the phenolic antioxidant ferulic acid (FA) to inhibit the neurological oxidative stress was demonstrated. The C/G/GP hydrogel ensures an excellent clinical expediency with a gelation temperature of 32.6 °C and gelation time of 75.58 s. In-vitro cytotoxicity assays of C/G/GP hydrogel and FA have revealed an excellent biocompatibility with the Neuro-2a cells. 500 μM of FA was considered to be an effective concentration to reduce the oxidative stress in Neuro-2a cells. TUNEL staining images evidenced that the H_2O_2 induced DNA fragmentation was comprehensively controlled after FA treatment. The mRNA gene expression profiles markedly authenticate the neuroprotectivity of FA by down-regulating ROS, inflammatory and apoptosis related markers. The outcomes of this study suggest that, C/G/GP hydrogel carrying ferulic acid could effectively protect further secondary traumatic brain injury associated impairments.

1. Introduction

Traumatic brain injury is a form of complex brain injury which may be either penetrating or non-penetrating/closed head trauma caused by an external mechanical force. According to the statistics of Department of Health, Taiwan, motor-vehicle accidents are ranked sixth among the top ten leading causes of death and more than 70% contributed to the intracranial hemorrhage that may lead to permanent brain damage or even death. During the years 2002–2007, approximately 1.7 million Americans sustained some kind of traumatic brain injury every year and it records 52,000 deaths and 124,000 disabilities [1]. TBI can be classified into mild, moderate and severe according to the magnitude of impact. The symptoms of TBI greatly varied with each form of TBI. A change in sleep patterns, behavioral or mood changes and trouble with memory, concentration, attention, or thinking are the common symptoms associated with moderate and severe TBI. In the case of mild TBI, the patient can experience a loss of consciousness for a few seconds or minutes along with the other physical symptoms like headache, nausea, fatigue and loss of balance.

The disruption of brain tissue due to a strong external mechanical force may contribute to primary traumatic brain injury [2]. The pathological consequences after primary TBI may include blood brain barrier (BBB) damage, contusion, diffuse axonal injury (DAI), hematoma, hypoxia/ischemia and an increase in intracranial pressure (ICP). These pathophysiological process are generally irreversible, however the primary injury significantly worsen the pathological conditions of brain damage by initiating the secondary injury immediately after the impact. The subsequent molecular and biochemical events initiated after the primary TBI, would constitute the secondary brain injury. The secondary injury is triggered by the release of excess of excitatory neurotransmitter, astrogliosis, neuronal depolarization, calcium influx, free radical generation, mitochondrial dysfunction, activation of caspases and proinflammatory cytokines [3]. The excitotoxicity imports the excessive production of reactive oxygen species and exhaustion of the endogenous antioxidant system (e.g. superoxide dismutase, glutathione peroxidase, and catalase) that significantly affects the normal cellular function by inducing the peroxidation of cellular and vascular structures, protein oxidation, cleavage of DNA and inhibition of the mitochondrial electron transport chain [4]. Excessive formation

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of highly reactive ROS such as superoxide ($O_2^{\bullet -}$), hydroxyl (•OH) and peroxyl (RO_2•) as well as non-radicals such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) elicited from secondary injury cascades has contributed to the massive neuronal death [5].

The recent research focus is towards the inhibition of secondary injury cascades for clinical intervention and targeted therapies. Inhibition of oxidative stress and neuronal inflammation is a promising strategy to treat central nervous system (CNS) related disorders and protect the brain from further tissue damage [6]. Different antioxidant approaches (enzymatic and non-enzymatic) have been attempted for the inhibition of oxidative stress [7,8]. Elevation of the antioxidant defense enzymes such as Cu/Zn superoxide dismutase, catalase (CAT), glutathione peroxidase (GPX) and glutathione-S-transferase (GST) is able to protect the neuronal cells from oxidative stress [9]. The antioxidant potential of propofol and erythropoietin (EPO) was tested to control the endogenous indices of oxidative stress in rat subjected to head trauma and concluded as an effective therapeutic method for brain injuries [10]. Various neuroprotective antioxidant compounds offer a significant reduction in post-traumatic brain damage in different experimental models with acute head injury [11]. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a natural phenolic compound found in many Chinese herbs like *Angelica sinensis*, *Cimicifuga heracleifolia* and *Lignsticum chuangxiong*, with excellent antioxidant, antimicrobial, anti-inflammatory, anticancer and neuroprotective effects [12].

The administration of antioxidants by oral, intraperitoneal and intravenous routes might decrease the free radical scavenging ability of the antioxidants due to long circulation time. Since most of the drug or pharmaceuticals are effusively active for shorter time and this limitation could be improved by conjugating them with suitable carriers [13]. Hydrogels are effective carrier system for the selective and sustained release of drug or active pharmaceuticals [14]. Injectable and physically cross-linked thermo-sensitive hydrogels are progressively used for numerous tissue engineering and drug delivery applications due to the absence of any toxic crosslinker and in vivo gelation [15]. The objective of this study is to evaluate the antioxidant and neuroprotective effects of ferulic acid release from chitosan/gelatin/glycerol phosphate (C/G/GP) hydrogel to treat H_2O_2 induced oxidative stress in Neuro-2a cells. As stated, oxidative stress is one of the key contributors for the progression of pathophysiological conditions associated with TBI. The toxicity of ferulic acid and C/G/GP hydrogel are extensively characterized by in vitro cytotoxic assays. The antioxidant and neuroprotective effects of ferulic acid treatment over the oxidative Neuro-2a cells are substantially demonstrated with the MitoSOX Red, TUNEL assay and gene expression profile by RT-PCR.

2. Experimental

2.1. Chemicals and reagents

Chitosan (molecular weight = 340 kDa, degree of deacetylation > 95%) was obtained from Kiotek, Taiwan. Gelatin (G1890), Acetic acid (33209) and β-Glycerophosphate disodium salt hydrate (G9422) were obtained from Sigma, USA. MitoSOX™ Red mitochondrial superoxide indicator (M36008) and High capacity cDNA Reverse Transcription Kits (4368813) were purchased from Invitrogen, USA. DeadEnd™ Fluorometric TUNEL System (G3250) and RNeasy® Mini Kit were respectively procured from Promega, USA and QIAGEN, Germany. The TaqMan® Universal Master Mix II was purchased from ABI, The other analytical chemicals or reagents were purchased from Sigma, USA unless otherwise specified.

2.2. Preparation of thermosensitive chitosan/gelatin/β-GP (C/G/GP) and C/G/GP–FA hydrogel

The thermosensitive hydrogel was prepared by 2.5% chitosan dissolved in 0.1 M acetic acid and 1% gelatin in water. The solutions were autoclaved for 30 min at 121 °C and the sterile chitosan and gelatin solution were mixed together under gentle stirring. Followed by the slow addition of β-Glycerophosphate disodium salt hydrate (β-GP, 44.4% dissolved in deionized water and filtered by 0.22 μm filter (Millex-GV, Millipore, USA) into chitosan/gelation solution under stirring condition. The final pH of the C/G/GP solution was adjusted to 7.4 and stored at 4 °C until further analysis. For C/G/GP–FA hydrogel preparation, 500 μM of ferulic acid (FA) dissolved in 0.1 M dimethyl sulfoxide (DMSO) was directly added to C/G/GP solution via pipetting.

2.3. Rheological characterization of C/G/GP hydrogel

A HAAKE RheoStress 600 rheometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with parallel plate geometry sensor (PP35 Ti, 35 mm diameter and 0.105 mm gap) in oscillatory mode was used to evaluate the rheological measurements of C/G/GP hydrogel. The rheological properties such as gelation temperature, gelation time and gel strength were measured. The gelation temperature was studied at a temperature range of 15 to 45 °C, with the gelation time of 10, 25 and 37 °C, respectively. The storage modulus (G′) and loss modulus (G″) were recorded at a frequency of 1.0 Hz and a constant stress of 5 Pa.

2.4. In vitro C/G/GP hydrogel degradation

The in vitro degradation of C/G/GP hydrogel was evaluated by transferring 100 μl C/G/GP solution to 2.0 ml micro-centrifuge tube and kept in an incubator at 37 °C until a stable gel is formed. In order to mimic the in vivo acidic environment after brain injury, C/G/GP hydrogel was soaked in 1.0 ml PBS (pH 6.0) that contains 2000 U lysozyme and placed in an incubator at 37 °C. The samples were incubated at different time intervals of 1, 2, 3, 5, 7, 14, 21 and 28 days to determine the degradation profile of C/G/GP hydrogel. After a predetermined time, the residual samples were separated from the solution and freeze-dried to determine the weight loss (W_d). The degradation percentage was calculated by the formula:

$$\text{Weight loss } (\%) = (W_i - W_d)/Wi \times 100$$

where, W_i is the initial weight of C/G/GP hydrogel before degradation and W_d is the final weight after degradation.

2.5. Encapsulation efficiency and release profile of ferulic acid

To calculate the encapsulation efficiency of ferulic acid, C/G/GP–FA hydrogel formulation containing 500 μM ferulic acid (1 ml) was dissolved in 0.1 M acetic acid (9.0 ml) and then subjected to a quick vortex (<10 s). Followed by, the intact ferulic acid concentration was determined by UV–Vis–NIR spectrophotometer (DU 7500, Beckman, USA) at a wavelength of 343 nm. The encapsulation efficiency of FA laden C/G/GP hydrogel was calculated according to Eq. (1).

$$\text{Encapsulation efficiency} = \text{Experimental FA loading}/\text{Theoretical FA loading} \times 100\%. \quad (1)$$

The release profile of ferulic acid (FA, 500 μM) from C/G/GP was assessed by transferring 200 μl of FA-hydrogel solution to 15 ml centrifuge tube and kept at 37 °C until complete gelation. Followed by, 2.0 ml of PBS was added into each tube (for different samples) and incubated again at 37 °C for a pre-determined time intervals of 0.5, 1, 2, 3, 4, 6, 12, 24 and 48 h. During the course of time, each tube was replenished with 2.0 ml of fresh PBS. The FA release from FA–C/G/GP gel during the afore-mentioned time intervals was recorded by an ultra violet–visible–near infrared (UV–VIS–NIR) spectrophotometer (DU 7500, Beckman, USA) at a wavelength of 343 nm. The linear calibration curve of FA release profile was constructed using the absorption value