Journal of Functional Foods 49 (2018) 250–257

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Water extract of Brazilian green propolis attenuates high glucose-induced vascular morphological abnormality in zebrafish

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ARTICLE INFO

Keywords:
Caffeoylquinic acid
Hyperglycemia
Propolis
Vascular morphology

ABSTRACT

In diabetes patients, hyperglycemia causes many vascular disorders. To clarify the effects of water extract of Brazilian green propolis (WEP) against high glucose-induced vascular disorders, we investigated using zebrafish-based vasculopathy model. Zebrafish larvae, which is transparent and small, is an ideal model organism to detect changes of the vascular structure. Zebrafish larvae were exposed to 200 mM glucose and WEP solution from 2 h post fertilization (hpf) to 3 days post fertilization (dpf). The WEP treatment normalized high glucose-induced morphological abnormality of the larvae, hyaloid vessels, and cerebral vessels. The WEP treatment partly suppressed the elevation of glucose levels and mRNA levels of p27 in tissues by exposure to high glucose content. Furthermore, we found that chlorogenic acid, which is one of the main constituents of WEP, also normalized high glucose-induced morphological abnormality. Our findings indicated that WEP improves vascular integrity in the central nervous system under high glucose conditions.

1. Introduction

Both acute and chronic hyperglycemia cause various malfunctions (Campos, 2015; Fiorentino, Prioletta, Zuo, & Folli, 2013; Xi, Shen, Wai, Vilas, & Clemmons, 2015), which are characterized as diabetes-related complications. These complications include microangiopathy (retinopathy, nephropathy and neuropathy) and macrovascular diseases (atherosclerosis, cardiovascular and cerebrovascular disorders) (Fowler, 2008). In this context, hyperglycemia closely relates with vasculopathy.

Propolis is one of the many bee products that are used as traditional medicine. The effects of propolis depend on the country of origin. Brazilian green propolis, that mainly originates from *Baccharis dracunculifolia*, is known for its potential against oxidative stress (Guimarães et al., 2012; Saito, Tsuruma, Ichihara, Shimazawa, & Hara, 2015), anti-inflammation (Lima et al., 2014; Wu et al., 2013), tumorigenesis (Frión-Herrera, Díaz-García, Ruiz-Fuentes, Rodríguez-Sánchez, & Sforcin, 2015), and angiostatic effects (Chikaraishi, Izuta, Shimazawa, Mishima, & Hara, 2010; Lima et al., 2014). Recently, two independent studies showed that Brazilian green propolis improves renal function and anti-oxidative activity in patients with type2 diabetes mellitus (Fukuda, Fukui, Tanaka, & Senmaru, 2015; Zhao et al., 2016). However, at present, it is unknown whether Brazilian green propolis has a protective effects against vascular disorders induced by hyperglycemia. The effects of propolis also depends on the solvent used for extraction. Previously, we confirmed that water extract of Brazilian green propolis (WEP) has therapeutic activity against oxidative stress and neovascularization (Chikaraishi et al., 2010; Saito et al., 2015). Here, we have investigated whether WEP protects vasculature under high glucose condition.

The vascular system is composed of several types of cells (vascular endothelial cells, smooth muscle cells, pericytes, astrocytes). In brain and retina, the vascular system is more complicated due to the presence of tissue-specific blood barriers (Hosoya & Tachikawa, 2013; Obermeier, Daneman, & Ransohoff, 2013). Therefore, a complete recapitulation of their vascular systems *in vitro* is difficult (Xie, Farage, Sugimoto, & Anand-Apte, 2010). We have thus focused on zebrafish (*Danio rerio*), whose larvae is an ideal model organism to investigate

Abbreviations: *p*-CA, *p*-coumaric acid; CGA, chlorogenic acid; MAPK, mitogen-activated protein kinase; NAC, N-acetyl cysteine; PTU, 1-phenyl-2-thiourea; qPCR, quantitative PCR; ROP, retinopathy of prematurity; SEM, standard error of the mean; SDS, sodium dodecyl sulfate; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; VEGF, vascular endothelial growth factor; WEP, water extract of Brazilian green propolis

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https://doi.org/10.1016/j.jff.2018.08.028
Received 20 May 2018; Received in revised form 31 July 2018; Accepted 18 August 2018

vascular morphology precisely since it has a transparent and small body that can be observed well with a microscope (Lieschke & Currie, 2007; Walcott & Peterson, 2014). Furthermore, zebrafish larvae also possesses the blood-brain and the blood-retinal barriers (Nishimura et al., 2005; Obermeier et al., 2013; Xie et al., 2010). In the present study, using zebrafish model, we have investigated that the effects of WEP against vascular disorders induced by high glucose levels.

We found that WEP suppressed the occurrence and severity of cerebral and retinal vascular morphological abnormality induced by treatment with high glucose in zebrafish larvae. However, WEP did not suppress abnormal angiogenesis in somite vessels. The WEP partly suppressed the abnormal elevation of tissue glucose levels in zebrafish larvae. However, it did not alter the abnormal elevation of lipid peroxidation levels in zebrafish larvae. Our experiments also revealed that chlorogenic acid contributes to the normalization of abnormal development. Our observation using zebrafish disclosed that WEP helps maintain normal development and vascular integrity under high glucose condition.

2. Material and methods

2.1. Water extract of Brazilian green propolis (WEP)

In the current study, the main source of Brazilian green propolis (Minas Gerais State, Brazil) was *Baccharis dracunculifolia*. Propolis was extracted using water at 50 °C to yield a water extract. The main constituents of WEP have been previously reported (Mishima et al., 2005). WEP used in this study mainly contained p-coumalic acid (p-CA), chlorogenic acid (CGA), 3,4-di-O-caffeoylquinic acid (3,4-CQA), 3,5-di-O-caffeoylquinic acid (3,5-CQA). Here, WEP, *p*-CA, and CGA were gifted by the API Co., Ltd., (Gifu, Japan).

2.2. Fish lines and maintenance

Zebrafish embryos and larvae of the Tg (fli1a: EGFP)^{y1} (from ZIRC) were produced by natural mating. The Tg zebrafish were established to visualize vascular structure (Lawson & Weinstein, 2002). In the Tg zebrafish, EGFP express under the fli1 promoter which is well known endothelial cell marker in zebrafish (Thompson et al., 1998). Embryos were raised at 28.5 °C in Danieau's solution under a 14 h light:10 h dark cycle according to previously described methods (Westerfield, 2007). All experiments were performed in accordance with the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) for the Use of Animals in Ophthalmic and Vision Research and were approved by Gifu Pharmaceutical University Committee on Use and Care of Animals.

2.3. Glucose and other chemical treatments

Roughly, 20 fertilized eggs were placed in 6-well plates with 4 mL Danieau's solution per well. Solutions were replaced every day. D-Glucose (Wako, Osaka, Japan), WEP, N-acetyl cysteine (NAC) (Wako), *p*-CA, CGA, and 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich, St. Louis, MO, USA), which is an inhibitor of melanin synthesis, were used as treatment after being diluted in the solutions.

2.4. Zebrafish morphology assay

Morphology of zebrafish larvae was observed using a BZ-X700 all-in-one fluorescence microscope (Keyence, Osaka, Japan). We observed zebrafish larvae directly that were placed in the 6-well plates without administering anesthesia.

2.5. In vivo imaging

All procedures were based on the methods of Y. Nishimura and T. Tanaka (Sasagawa et al., 2016). Zebrafish were anesthetized with 500 ppm 2-phenoxyethanol (Wako), and transferred onto 35-mm glass bottom dishes (Greiner Bio-One International GmbH, Kremsmuenster, Austria). A few drops of 3% low-melting agarose (Sigma-Aldrich) were laid over the living larvae, which were immediately oriented on the lateral side (to obtain images of hyaloid vessels and somite vessels) or upside down (to obtain images of cerebral vessels). Hyaloid, somite, and cerebral vessels of the embedded larvae were observed using a Zeiss LSM700 confocal laser scanning microscope (Carl Zeiss, Jena, Germany). We processed the z-stacked images to obtain maximum projection using Fiji (Schindelin et al., 2013).

2.6. Evaluation of tissue glucose levels in zebrafish larvae

Euthanization of 3 dpf zebrafish larvae by putting them into ice-cold fish water for 5 min. Just after euthanization, twenty zebrafish larvae in each experimental group were homogenized with 500 μL deionized water. Tissue glucose levels were evaluated using a glucose (GO) assay kit (Sigma, USA). A total of 1 mL of the assay reagent was added and the content were incubated for 30 min at 37 °C. Fluorescence was measured at 560 nm by scanning with a microplate reader (GloMax-Multi Detection System; Promega, Madison, WI, USA).

2.7. Quantification of tissue thiobarbituric acid-reactive substances (TBARS) levels in zebrafish larvae

Euthanization of 3 dpf zebrafish larvae by putting them into ice-cold fish water for 5 min. Just after euthanization, twenty zebrafish larvae from each experimental group were sonicated with 400 μL of 1.15% ice-cold potassium chloride solution. The larvae were then homogenized and centrifuged at 3,000 rpm and 4 °C for 10 min. A 200 μL aliquot of the supernatant was heated with 20 μL of 8.1% sodium dodecyl sulfate (SDS) solution and 200 μL of acetic acid buffer (pH 3.6) containing 0.8% of thiobarbituric acid (TBA) solution at 100 °C for an hour. After cooling, 400 μL of butyl alcohol and pyridine (15:1) mixture was added and the contents were centrifuged at 4000 rpm for 10 min. The butyl alcohol-pyridine phase containing the TBARS was then separated and its absorbance was measured at 532 nm with a microplate reader (Varioskan Flash 2.4; Thermo Fisher Scientific, Waltham, MA, USA).

2.8. RNA isolation

Euthanization of 3 dpf zebrafish larvae by putting them into ice-cold fish water for 5 min. Just after euthanization, twenty zebrafish larvae from each experimental group were sonicated with 350 μL of cell lysis buffer contained 1% 2-mercaptoethanol. RNA was isolated from the lysate with a NucleoSpin® RNA kit (Takara, Shiga, Japan) according to the manufacturer's protocol. The RNA concentrations were determined spectrophotometrically at 260 nm by NanoVue Plus (GE Healthcare Japan, Tokyo, Japan). The isolated RNAs were converted into first-strand cDNA using a PrimeScript RT reagent kit (Perfect Real Time; Takara) according to the manufacturer's protocol.

2.9. Quantitative PCR (qPCR)

The level of expression of the mRNAs of the p27 genes was determined by quantitative real-time-PCR (qRT-PCR). SYBR Premix Ex TaqTM II (Takara) and a TP 8000 Thermal Cycler Dice Real Time system (Takara) were used. The PCR primer sequences were as follows:

P27;
F 5′-TGAAGCCTGGAACTTCGACT-3′
R 5′-TGTGAATATCGGAGCCCTTC-3′
Glyceraldehyde-3-phosphate dehydrogenase (Gapdh);
F 5′-ATGACCCCTCCAGCATGA-3′
R 5′-GGCGGTGTAGGCATGAAC-3′.