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Mitigation of stress from gastric mucosal injuries by mulberry extract may occur via nitric oxide synthase signaling in mice

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ABSTRACT

Acute gastric mucosal injuries are serious clinical problems worldwide and are principally found with different types of stresses in animals. A constant challenge is to find original plant products that can combat stress. In the present study, we examined the effects of big-leaf mulberry extracts on stomach injury, and the activity of nitric oxide synthases (NOS) and total antioxidant activity (TAO) in the gastric mucosae of mice during water immersion and restraint stress (WIRS). Our data showed that WIRS-exposed mice produced several injuries and showed an enhanced iNOS, reduced eNOS activity, and decreased TAO activity in the stomach, whereas pretreatment with big-leaf mulberry extracts increased TAO activitiy. The data from our immunohistochemical study indicated that both iNOS and eNOS were expressed in parietal cells and blood vessels, while nNOS was only weakly expressed in parietal cells. In conclusion, our findings suggested that big-leaf mulberry mitigated WIRS-induced stomach injuries, and NOS signaling may play important roles in the mouse stomach during the recovery process.

1. Introduction

Acute gastric mucosal injuries are serious clinical problems worldwide, and may even lead to gastric cancer (Park et al., 2011). In mammals, stress can induce acute erosion of the gastric mucosa (Moody et al., 1976); and could decrease blood flow to the stomach (Kitagawa et al., 1979); increase oxidative stress; and change gene expression of stomach genes such as increasing inducible nitric oxide synthase (iNOS) mRNA expression and decreasing constitutive nitric oxide synthase (cNOS) mRNA expression (Szlachcic et al., 2013). However, the exact mechanisms involved in these changes remain unclear.

Nitric oxide (NO) is a free radical and highly reactive lipophilic molecule that exists in tissues and organs, and participates in a variety of biologic actions under both physiologic and pathologic conditions (Fedail et al., 2013; Dellamea et al., 2014). Three distinct isoforms of NOS have been identified in mammals: nNOS, iNOS, and eNOS; all are Ca^{2+}-dependent enzymes (Xu et al., 2013), and nNOS and eNOS are recognized as constitutive NOS (cNOS). Researchers have indicated that NO plays a biphasic role in the ulcerogenic response of the gastric mucosa (Ribbons et al., 1995; Tanaka et al., 1999); and that low concentration of NO–produced by constitutive eNOS–increases blood flow to help wound healing (Hou et al., 1999) in gastric mucosal injury. eNOS also regulate vascular homeostasis in endothelial cells (Nishida et al., 1992). However, the enhanced generation of NO by iNOS may unfortunately contribute to the pathogenesis of various inflammatory processes, including peptic ulcers (Jaiswal et al., 2001; Souza et al., 2004).

Investigations of plant extracts that combat stress are challenging. Many plant extracts have been proven to alleviate stress, *e.g.*, extracts of *Curcuma xanthorrhiza* leaf (Rahim et al., 2014), Banhabaekchulchunmatang (Shin et al., 2013), Lion's Mane Mushroom *Hericium erinaceus* (Wong et al., 2013), and Rutin (Mehfooz et al., 2018). As a new hybrid plant, big-leaf mulberry has proven to play a significant role in stress-induced testicular injury (Xu et al., 2014). Like other phytochemical plants, mulberry can be used to treat several diseases (Ramaa et al., 2006). It has been reported that mulberry attenuates oxidative stress (Hajizadeh et al., 2014), ameliorates obesity-related inflammation (Lim et al., 2013), exerts anti-hyperglycemic effects (Nazari et al., 2013), and increases apoptosis of cancer cells by reducing the NO produced by iNOS (Deepa et al., 2013).

Water immersion and restraint stress (WIRS) was first found by Takagi, and was widely used in the laboratory as a model to investigate gastric mucosal lesions (Nie et al., 2003; Landeira-Fernandez, 2004; Huang et al., 2012, 2016). Our previous work indicated that big-leaf

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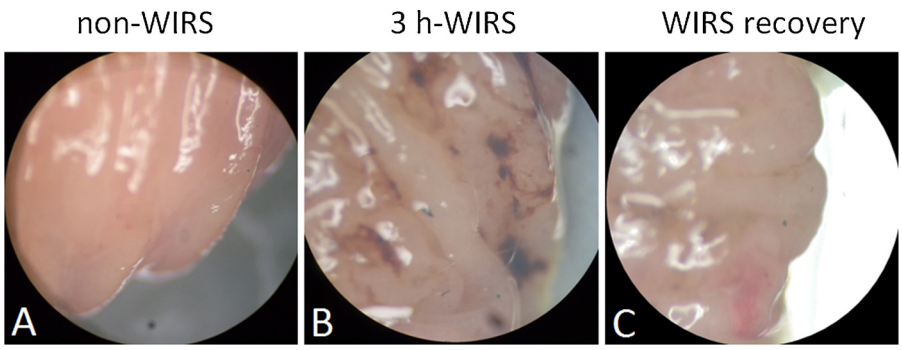

Fig. 1. Gross comparative observations of the gastric mucosa in the stomachs of mice. (A) The non-WIRS group showed normal gastric mucosa with no damage; (B) the 3 h-WIRS treated groups showed hemorrhages, errosions, and inflammatory injuries; (C) the WIRS recovery group showed that the mucosae were almost healed after a 10- day recovery period. The photographs were captured at 10× magnification with an inverted biologic microscope (optec BDS200).

mulberry exerts protective effects against stress-induced testicular injury (Xu et al., 2014); however, these data were found lacking due to the effects of big-leaf mulberry on WIRS-induced gastric lesions. In the present investigation, we used the WIRS model to study the roles of big-leaf mulberry extracts in gastric lesions, and examined NOS subunit expression and TAO activity in mouse stomach.

2. Materials and methods

2.1. Big-leaf mulberry plants

Big-leaf mulberry is a new hybrid plant arising from cell fusion and plant tissue technology from 2 families of deciduous trees, and shows high nutritional value to the food industry (Fig. 1S). Leaves of big-leaf mulberry plants were collected from a local area (Quzhou, Zhejiang, China). The crude protein, crude fiber, neutral detergent fiber (NDF), acid detergent fiber (ADF), hemicellulose, and crude ash of the big-leaf mulberry plant leaves used in the present project were 18.71%, 16.28%, 21.81%, 5.54% and 9.42%, respectively. We placed 15 g of dried mulberry leaves in 500 ml of water and boiled them for 20 min; then the leachate was collected and cooled. This extracted mulberry solution was used fresh for consumption by the experimental mice.

2.2. Animals

Thirty male Swiss ICR (Institute for Cancer Research) mice 2 months of age were bought from Qinglongshan Laboratory Animal Company (Nanjing, China). All animals were kept in an animal room and randomly divided into 2 major groups, 1 treated and the other served as controls. The mice of the control group had free access to tap water while the treated mice had individual free access to the extracted big-leaf mulberry solution, and both groups were fed a mouse diet *ad libitum* throughout the 1-month study period. We further randomly divided each group into 3 subgroups as follows: control mice (n = 5) without exposure to WIRS and a treated subgroup of mice (n = 10) were fixed in a restraint device and immersed in a water bath (18 °C) up to their xiphoid processes for 3 h (WIRS group) (Adachi et al., 2011). Treated animals were sacrificed at 2 different times: immediately after the 3-h WIRS treatment or 10 days later. Before starting the experiment, we deprived all mice of food for 24 h, but water was allowed. Stomachs were collected and initially photographs of the samples were captured at a 10-fold magnifications under an inverted biologic microscope (optec BDS200); and some portions of the stomachs were stored at −80 °C, while others were fixed in 4% paraformaldehyde (PFA) overnight for histo-pathologic and immunohistochemical analyses. All experiments were performed according the guidelines of the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Nanjing Agricultural University, China. Permission to use laboratory animals in our university was certified as No. SYXK(Su) 2017-0007, and the ethics approval number of this project was NAU2015018 from our university ethics committee.

2.3. Determination of stomach T-AOC levels and NOS activity

TAO levels and NOS activity were determined by commercial reagents (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); optical density was detected using an ELISA reader (Bio-TEK Instruments, Winooski, VT, USA), and the process was in strict accordance with the manufacturer's protocols. Tissues were weighed and homogenized in iced saline to create a 1:10 suspension. After centrifugation at 2500 r for 10 min at 4 °C, TAO levels were measured at 520 nm by the ferric-reducing ability of plasma (FRAP) assay and the NOS activity was determined based on the release of local NO generated from a 5-electron oxidation of terminal guanidinium nitrogen on L-arginine by NOS, and measured at 530 nm. Data were expressed as U/mg protein.

2.4. Histo-pathologic analysis

The fixed stomach samples were dehydrated in a graded series of alcohols, cleared in xylene, and then embedded in paraffin. Tissues were sectioned serially at 6 μm and slides in every group were stained with hematoxylin and eosin (H&E), and observed under a light microscope (Nikon, Tokyo, Japan).

2.5. Immunohistochemistry

To study the cellular localization of nNOS, iNOS, and eNOS (purchased from Boster Biological Technology, Wuhan, China) in the stomachs of mice, and sections were cut at 6 μm. Following deparaffinization and hydration, tissues were heated in 0.01 mol/l citrate buffer for 10 min in a microwave oven. The endogenous peroxidase activity and nonspecific binding were blocked with H_2O_2 in methanol for 1 h and bovine serum albumin (BSA) for 2 h. Sections were incubated overnight at 4 °C with antibodies to nNOS, iNOS, and eNOS (diluted at 1:200 in PBS). Finally, the slides were incubated with diaminobenzidine substrate (DAB; Sigma-Aldrich, St. Louis, MO, USA) and nuclei were stained with hematoxylin. We incubated negative control tissues with normal rabbit serum (NRS) instead of the primary antibody.

2.6. Statistical analysis

All values are expressed as means ± SEM. Our statistical analysis was performed using 1-way analysis of variance (ANOVA) followed by Fisher's protected least significance (PLSD) test for multiple comparisons, and Student's *t*-test for single comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Gross observations

Gross findings of the gastric mucosae (Fig. 1) showed that stomachs

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