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Efficacy of Zhuling polyporus polysaccharide with BCG to inhibit bladder carcinoma

Guo-wei Zhang[a,*], Gui-fang Qin[a], Bo Han[a], Cai-xia Li[b], Hong-Gai Yang[a], Pi-hu Nie[a], Xing Zeng[c,*]

[a] College of Chinese Medicine, Hebei University, Baoding 071002, China
[b] The School of Basic Medical Science, Guangzhou University of Chinese Medicine, Guangzhou 510006, Guangdong Province, China
[c] Guangdong Provincial Academy of Chinese Medical Sciences, Guangdong Provincial Traditional Chinese Medicine Hospital, Guangzhou 510006, Guangdong Province, China

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ABSTRACT

There is growing interest in reducing Bacille Calmette-Guerin (BCG) side effects while keeping intact its therapeutic efficacy. In the present study, we evaluated the efficacy of Sclerotia of *Polyporus umbellatus FRIES* (Zhuling) and its main ingredient Polyporus Polysaccharide (PPS) to attenuate side effects of BCG therapy in vivo. The results show that bladder cancer development in model rats exhibited significantly reduced cancer invasiveness with Zhuling PPS combined with BCG. Flow cytometric (FCM) analysis showed expression of costimulatory molecules CD86, CD40, and TLR4/CD14 significantly increased with Zhuling PPS in combination with BCG. Similarly, immunohistochemical analysis revealed stronger CD86 and CD40 staining. Our findings show Zhuling PPS strongly reduced side effects and displayed synergistic effects during BCG instillation in rat bladder cancer models. The findings also suggest that the attenuation effect may result from direct activation of dendritic cell (DC) TLR4.

1. Introduction

Bladder cancer is the fourth most common malignancy in the Western world, following prostate, lung, and colon cancer. In 2014, it is estimated that 74,690 Americans will be diagnosed with bladder cancer and 15,580 will die (Siegel, Ma, Zou, & Jemal, 2014). More than 90% of bladder cancers are transitional cell carcinoma (TCC). Immunotherapy with Bacillus Calmette-Guerin (BCG) instillation is recommended for high-risk, non-muscle invasive bladder cancer and recurrence prevention, and as a supplement to transurethral resection (TUR). However, the duration of BCG instillation causes severe local and systemic side effects such as cystitis and hematuresis. Thus, it is important to find new methods or drugs to reduce side effects that do not compromise its therapeutic effect.

Naturally occurring substances, such as those found in traditional Chinese medicine, are promising candidates (Khan, Afaq, & Mukhtar, 2008). Zhuling Sclerotia of *Polyporus umbellatus FRIES* (Zhuling) is used widely in Traditional Chinese medicine and has a diuretic effect according to work by Shen Nong Ben Cao Jing. Polyporus polysaccharide (PPS) has been identified as the primary active substance in Sclerotia and has been shown to have anticancer efficacy against bladder cancer both in vitro and in vivo (Wei, Zeng, Han, & Huang, 2011, Zhang et al., 2011).

The host immune system is often considered defective, allowing malignant cells to evade host antitumor defenses. In addition, it has been shown that patients with bladder cancer have a dysfunctional immune system (Agarwal, Agrawal, Verma, Mohanty, & Saxena, 2010). Notably, previous reports have shown that PPS inhibits BCG induction of the NF-κB signaling pathway in bladder cancer. However, to the best of our knowledge, there are no reports that Zhuling and PPS attenuate adverse reactions to BCG therapy in vivo (Wei et al., 2011; Zhang et al., 2011). In the present report, we show that Zhuling and PPS possess anticancer activity when combined with BCG and used to treat bladder carcinoma, and propose a preliminary molecular mechanism of attenuation.

2. Materials and methods

2.1. Experimental animals

Female Fisher-344 rats were purchased from Vital River Laboratories (Beijing, China), and used after 3 d acclimation. All animals

* Corresponding authors at: Hebei University, College of Chinese Medicine, Number 342 Road, Nanshi District, Baoding 071002, China. Tel.: +86 20 39318678/ +86 312 5078515.
E-mail addresses: xxzgw@126.com, guoweizhang2009@gmail.com (G.-w. Zhang), zengxing-china@163.com (X. Zeng).

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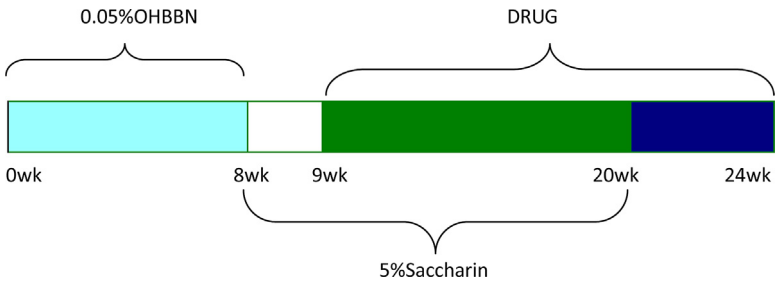

Fig. 1. Eight-week-old female F344 rats were randomly divided into five groups. Bladder cancer was induced in groups 2–5 by administration of BBN (0.05%, W/V) in drinking water for 8 weeks, then given Saccharin (5%, W/V) in their drinking water through to 20 weeks. Drinking water containing carcinogen was changed twice a week. Rats in groups 1, 2, and 5 were gavaged with 0.9% NaCl. Rats in groups 3 and 4 were gavaged with Zhuling and PPS. Rats in groups 3, 4, and 5 were given BCG by intravesical instillation each week from 9 to 15 wk, and from 19 to 23 wk.
Group 1 (10 rats): 0.9% NaCl, Group 2 (10 rats): BBN + 0.9% NaCl, Group 3 (10 rats): BBN + BCG (2 mg/rat/week) + Zhuling (250 mg/kg body weigh/d), Group 4 (10 rats): BBN + BCG (2 mg/rat/week) + PPS (28 mg/kg body weigh/d), Group 5 (10 rats): BBN + BCG (2 mg/rat/week).

were handled following the Guiding Principles for the Care and Use of Experimental Animals from Guangdong provincial TCM Hospital, and approved by the institutional committee on animal care. All animals were maintained under standard environmental conditions (23 ± 2 °C, 55 ± 5% humidity and 12 h/12 h light/dark cycle). All animals were allowed free access to tap water and standard laboratory rat food.

2.2. Materials

Zhuling was purchased from Tianjiang Pharmaceuticals Limited (Jiang Yin, China). Zhuling was mixed with water 1:10 (g/mL), and the mixture boiled at 100 °C under reflux for 30 min. The substance obtained was centrifuged, filtered, concentrated, and then sprayed for drying, yielding approximately 5% (w/w) of dry Zhuling extract. The product was dissolved in water (25 mg/mL) before administrated at the stated doses. PPS was extracted from Zhuling and its average molecular weight number (*Mn*) calculated to be 48,232, average molecular weight (*Mw*) of 117,506, and polydispersity (*Mw*/*Mn*) of 2.44, and was composed of glucose (62.28%) and trehalose (6.05%). PPS was dissolved in water (2.8 mg/mL) before administration at the stated doses. The Zhuling-derived PPS was 869 mg/g as determined by the anthrone-sulfuric acid method.

2.3. Experimental design

Rats were randomly assigned to five groups (Fig. 1). After 3 d acclimation, rats in groups 2–5 were given 0.05% BBN (W/V)(TCI, Tokyo, Japan) drinking water for 8 weeks to induce bladder cancer, followed by 5% saccharin until week 20, according to published protocols (Nakanowatari, Fukushima, Imaida, Ito, & Nagase, 1988; Kakizoe, Komatsu, Niijima, Kawachi, & Sugimura, 1981). Daily oral doses of Zhuling extract and PPS were administered to groups 3 and 4 from week 9 through to week 20. The volume of each gavage was 1.0 mL/100 g and given daily (7×/week). BCG (60 mg/3 mL), dissolved with physiologic saline, was given intravesically to groups 3, 4, and 5 from 9 wk to 15 wk and then 19 wk to 23 wk. The volume of each administration was 0.1 mL/100 g and was given weekly.

The rats were observed daily for signs of toxicity, weighed weekly, and palpated for urinary bladder lesions twice weekly. To assess food intake, rats were weighed 2 weeks before the end of the experiment, and bladder, spleen, and thymus indices determined. At necropsy, urinary bladders were weighed and then half inflated with 10% buffered formalin and the other half bladders immediately frozen in liquid nitrogen at −80 °C until measurements were taken. After fixation, bladders were observed under a high intensity light for gross lesions, and each lesion dissected and processed (H&E-stained) for histological classification.

2.4. Flow cytometer analysis (FCM)

Peritoneal macrophages were collected from the peritoneal cavity by lavage of sacrificed rats. The percent phagocytosis and expression of co-stimulatory surface markers CD86, CD40, and TLR4/CD14 were detected by FCM and analyzed using CXP software (Analis, Saurlee, Belgium).

2.5. Immunohistochemical analysis of CD86 and CD40

Tumor tissues were fixed in 10% ethanol and embedded in paraffin. Following deparaffinization, samples were heated in unmasking solution at 95 °C for 15 min. Nonspecific binding sites were blocked with goat serum. Sections were then incubated overnight at 4 °C in a humidified chamber with the following primary antibodies: rabbit anti-CD86 (1:200), or rabbit anti-CD40 (1:250). All dilutions were in 0.5% BSA in PBS. Sections were incubated with the appropriate secondary at a 1:500 dilution for 30 min. Protein staining was visualized by incubating sections with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO, USA) and lightly counterstaining with hematoxylin. Photographs were obtained using an Eclipse E microscope digital camera (Nikon, Tokyo, Japan).

3. Statistical analysis

Results are expressed as mean ± SD. Statistically significant differences were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test (SPSS 11.5, IBM, Chicago, IL, USA). A $p < 0.05$ was considered statistically significant.

4. Results

4.1. General observations and histological examination

The mean body weights of BBN, BBN + BCG + Zhuling, BBN + PPS, and BBN + BCG groups did not differ compared with the control group (Fig. 2A). Some rats did die during the experimental period after receiving BCG, although no rats given PPS died (Fig. 2B). There were also no differences in food intake between rats in experiment groups compared with the control group (Fig. 2C). The average bladder, spleen, and thymus weights after adjustment by body weight (mg tissue weight per 1 g body weight) were determined for all groups. The dosing regimen used in this study (0.05% BBN in