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Efficacy of Zhuling polyporus polysaccharide with BCG to inhibit bladder carcinoma

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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

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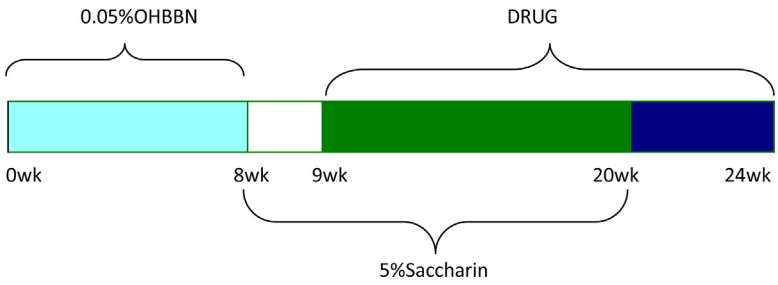


Fig. 1. Eight-week-old female F344 rats were randomly divided into five groups. Bladder cancer was induced in groups 2–5 by administration of BBN (0.05%, W/V) in drinking water for 8 weeks, then given Saccharin (5%, W/V) in their drinking water through to 20 weeks. Drinking water containing carcinogen 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

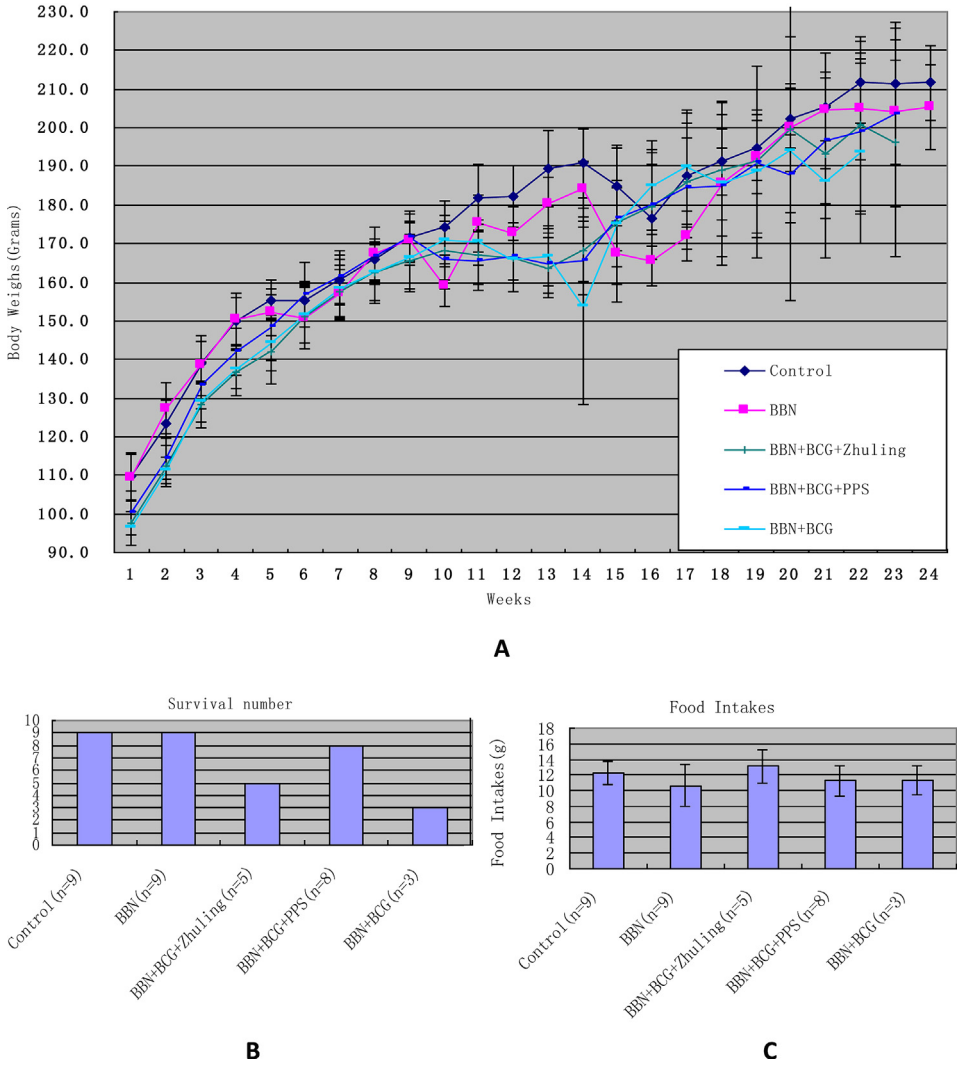
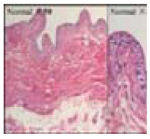
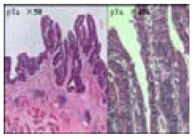
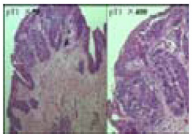
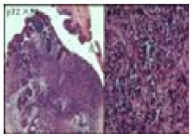


Fig. 2. (A) Body weight of each rat was recorded weekly throughout the experiment and mean body weight of all rats was plotted as a function of each week for each group. (B) Survival number was recorded when the experiment ended. (C) Food intake. Values are expressed as mean ± S.D. Rats in each group, * $P < 0.05$, ** $P < 0.01$, compared with controls using Dunnett's *t*-test.

drinking water for 8 weeks and 5% saccharin in drinking water for 12 weeks) was highly effective in inducing bladder cancer (Zhang et al., 2011). Whereas no rats in the control group developed bladder cancer, 8/9 BBN-Saccharin-treated rats did. In addition, the remaining rat had dysplasia in the bladder, 3 BBN rats had bladder cancer localized within the epithelium (pTa), and 1 rat presented submucosal (pT1) and 4 muscular (pT2) invasion. By treating with Zhuling or PPS combined with BCG, bladder cancer development was inhibited and displayed reduced invasiveness (Table 1). The bladder index results indicated that the acute bladder weight increase was due to PPS feeding combined with BCG, which was observed in the short-term and was completely reversible

Table 1
Morphology bladder cancer stage in each group.

Group	Number	Normal	Dysplasia	pTa	pT1	pT2
						
Control	$n=9$	9	0	0	0	0
BBN	$n=9$	0	1	3	1	4
BBN + BCG + Zhuling	$n=5$	0	2	3	0	0
BBN + BCG + PPS	$n=8$	0	4	4	0	0
BBN + BCG	$n=3$	0	2	1	0	0

Microscopic images were obtained after H&E staining of paraffin-embedded bladder tissues. pTa, pT1, and pT2 refer to noninvasive superficial tumors, invading the lamina propria, and invading the muscularis propria.

Table 2
Relative bladder, spleen, and thymus weights of rats in each group.

Group	Number	Value (mg/g, mean ± S.D)		
		Bladder index	Spleen index	Thymus index
Control	$n=9$	0.59 ± 0.29	1.89 ± 0.18	1.17 ± 0.30
BBN	$n=9$	1.00 ± 0.96**	2.08 ± 0.22*	1.19 ± 0.57
BBN + BCG + Zhuling	$n=5$	2.48 ± 2.13*,Δ	1.84 ± 0.98	0.70 ± 0.43*,Δ
BBN + BCG + PPS	$n=8$	0.98 ± 0.68	2.10 ± 0.64	1.08 ± 0.43
BBN + BCG	$n=3$	0.86 ± 0.26	1.81 ± 0.83	0.88 ± 0.43

Values are expressed as mean ± S.D. of rats in each group.
* $P<0.05$.
** $P<0.01$, compared to controls using Dunnett's t-test.
Δ $P<0.05$, compared with BBN group using Dunnett's t-test.

Table 3
Expression of co-stimulatory molecules CD86, CD40, and TLR4/CD14 on rat peritoneal macrophages in each group.

Group	Number	CD86 (%)	CD40 (%)	TLR4/CD14
Control	$n=9$	35.1 ± 5.94	3.63 ± 1.28	19.7 ± 8.16
BBN	$n=9$	10.70 ± 5.37*	0.87 ± 0.38*	32.85 ± 5.09*
BBN + BCG + Zhuling	$n=5$	15.65 ± 2.03*	2.30 ± 1.40*	47.9 ± 5.90Δ
BBN + BCG + PPS	$n=8$	18.02 ± 2.98*Δ	9.66 ± 2.14*Δ	27.53 ± 5.49*
BBN + BCG	$n=3$	14.6 ± 2.5*	1.03 ± 0.20*	54.15 ± 3.40Δ

Values are expressed as mean ± S.D. of rats in each group. Statistical analyses of CD86, CD40, and TLR4/CD14 data was compared to control group using Dunnett's t-test.
* $P<0.05$.
** $P<0.01$, compared with control group using Dunnett's t-test.
Δ $P<0.05$, compared with BBN group using Dunnett's t-test.

(Table 2). The spleen and thymus indices indicated the Zhuling aqueous extract and PPS seemed to offer effective immunoprotection (Table 2).

4.2. CD86, CD40, and TLR4/CD14 expression in peritoneal macrophages and bladder epithelial cells

FCM analysis revealed expression of costimulatory molecules CD86, CD40, and TLR4/CD14 increased with BCG treatment or in combination with Zhuling and PPS (Table 3, Fig. 3). Interestingly, immunohistochemical analysis showed CD86 expression was detected mainly in epithelial cells near cancerous tissue, but did not appear to be expressed within cancerous cells. CD40 was highly expressed in transitional bladder cancer, and significant differences in CD40 staining was observed between normal bladder and transitional bladder cancer cells, even when exhibiting stronger CD86 and CD40 expression (Fig. 4).

5. Discussion

PPS extracted from Zhuling has traditionally been used as a diuretic, and has shown promise as an adjuvant alongside chemotherapy. It has been licensed in China and has been reported to improve treatment outcomes and quality of life indicators in patients with a number of cancers including lung, liver, and leukemia (Chang & But, 1986; Zhu, 1987). Our results indicate that Zhuling PPS is highly effective in inhibiting bladder carcinogenesis in rats (Wei et al., 2011). The pharmacological inhibition of bladder carcinogenesis is mediated via the immune system, including TLR4 mediated macrophage activation and increased antibody production, as indicated through in-vitro and in-vivo studies (Li & Xu, 2011; Li, Xu, & Chen, 2010).

Dendritic cells (DCs) play a central role in inducing antitumor immune responses. Induction of an effective antitumor immune response requires the activation of effector cells by antigen-presenting cells (APCs) responsible for the presentation of tumor specific antigens. DCs are highly effective APCs that directly activate naive T cells. At present, DC-based therapeutic vaccines are being tested in clinical trials (Banchereau et al., 2005). Indeed, experimental models have demonstrated tumor rejection by autologous hosts and shown to be mediated most effectively by T cells (Hart & Colaco, 1997). Activation of tumor-specific T cells requires the processing and presentation of tumor antigen by professional APCs (Ostrand-Rosenberg, 1994).

Studies using a number of experimental models have shown that tumor cells engineered to express CD86 elicit an effective anti-tumor T cell response (Allison, Hurwitz, & Leach, 1995). CD86 is the major ligand for CD28, and expression of these ligands is

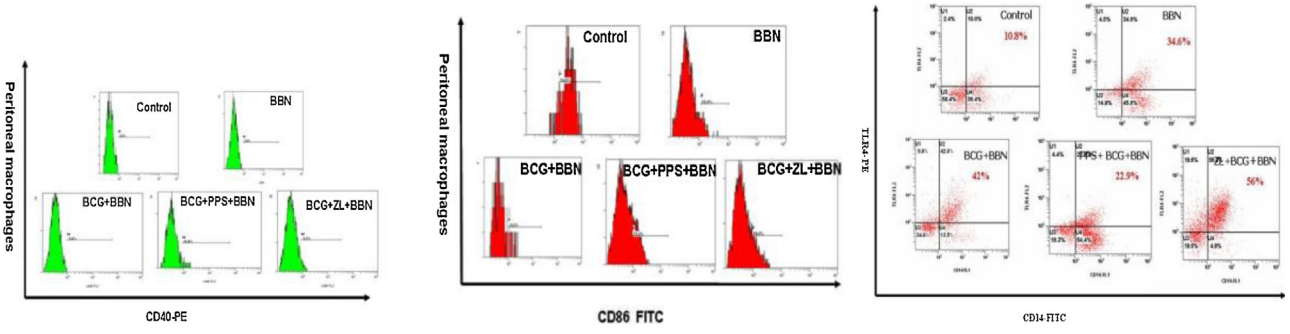


Fig. 3. Expression of CD86, CD40, and TLR4/CD14 of peritoneal macrophages in each group.

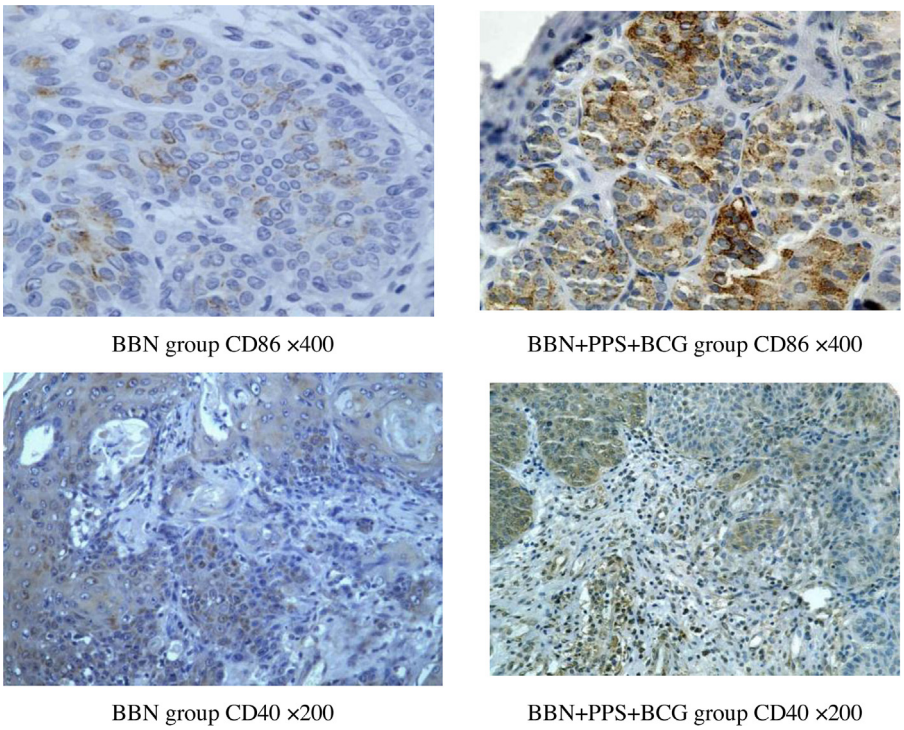


Fig. 4. Immunohistochemical analysis on CD86 and CD40 in the cellular membrane and bladder tissue endochylema.

generally limited to professional APCs, but exhibit limited expression in recurrent bladder cancer (Dallman, Johnson, & Packham, 2003). Previous research showed that the number of circulating myeloid DCs (mDCs) decreases and that CD86 expression levels are lower in bladder cancer patients than in healthy controls (Lang, Linlin, Ye, & Yuhai, 2012).

The slight proliferative response may be attributed to the presence of other costimulatory molecules, such as CD40 (Grewal et al., 1996). CD40 is a surface receptor best known for its capacity to initiate multifaceted activation signals in normal B cells and DCs. CD40-mediated interactions play an important role in the response to infections and cancer by affecting the development, activation, proliferation, and differentiation of a variety of immune cells (Alexandroff et al., 2000). Abnormal CD40 signaling seems to be directly associated with the pathogenesis of chronic inflammatory diseases associated with cancer (Feng et al., 2012). CD40-mediated signal transduction may be a new therapeutic agent through strategies leading to blockage of specific CD40-induced responses (Dallman et al., 2003).

There is a high recurrence rate of bladder carcinoma even after surgery. Intravesical instillation of BCG in transitional cell carcinomas (TCC) of the urinary bladder is an example of effective immunotherapy (Martínez-Piñeiro & Martínez-Piñeiro, 1997). However, BCG immunotherapy is accompanied by significant side effects that limit its clinical application. Local inflammatory responses up-regulate MHC class II expression and recruit T helper cells and mononuclear phagocytes (Prescott, James, Hargreave, Chisholm, & Smyth, 1992). It is known that the production of several inflammatory associated molecules is associated with BCG therapy (Jackson et al., 1998). BCG binds to the urothelium and is internalized by bladder cancer cells that express BCG surface antigens (Dallman et al., 2003).

The mechanism of intravesical BCG has not been fully elucidated, though it appears to involve induction of a T cell response, and BCG acts mainly as a potent immune adjuvant for TLR4 activation of NF-κB (Kim, Kim, & Choi, 2013).

We did not observe any rats in the control group that developed bladder cancer. However, 8/9 BBN-Saccharin-treated rats developed bladder cancer under the dosing regimen used, and the remaining rat exhibiting dysplasia in the bladder. Three rats in the BBN group had bladder cancer localized within the epithelium (pTa), and one rat presenting with submucosal (pT1) and four with muscular (pT2) invasion. Together, these results indicate that bladder carcinoma was successfully treated in the rat model used. Bladder cancer development was significantly inhibited and exhibited reduced invasiveness in rats treated with Zhuling PPS combined with BCG. Bladder index results indicate that acute bladder weight increases due to PPS feeding combined with intravesical administration of BCG, and single BCG side effects were completely reversible in the short-term. Thus, the spleen and thymus indices suggest that Zhuling aqueous extract and PPS may provide effective immunoprotection.

FCM analysis showed expression of costimulatory molecules CD86, CD40, and TLR4/CD14 increased with administration of BCG or the combination of Zhuling and PPS. Immunohistochemical analysis showed CD86 expression was detected mainly in epithelial cells near cancerous tissue, with little or none expressed in cancerous cells. CD40 was highly expressed in transitional bladder cancer cells, with significant differences in CD40 staining observed between normal and transitional bladder cancer, where CD86 and CD40 expression was strongest.

6. Conclusion

These findings suggest Zhuling and PPS attenuate the efficacy of BCG instillation in rat bladder cancer. These findings also infer that the attenuation effect may directly activate the DC TLR4 response. In summary, our results provide experimental evidence to support PPS as a strong attenuation candidate for BCG instillation against bladder carcinoma.

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