Comparison of intraperitoneal anti-adhesive polysaccharides derived from *Phellinus* mushrooms in a rat peritonitis model

Jae-Sung Bae, Kwang-Ho Jang, Hee-Kyung Jin

**Abstract**

**AIM:** To assess the adhesion- and abscess-reducing capacities of various concentrations of polysaccharides derived from fungus, *Phellinus gilvus* (PG) or *Phellinus linteus* (PL) in a rat peritonitis model.

**METHODS:** In 96 SD rats, experimental peritonitis was induced using the cecal ligation and puncture model (CLP). Rats were randomly assigned to 8 groups; Ringer’s lactate solution (RL group), hyaluronic acid (HA group), 0.025%, 0.25%, and 0.5% polysaccharides from PG (PG0.025, 0.25, 0.5), and PL (PL0.025, 0.25, and 0.5) groups, and PL (PL0.025, 0.25, and 0.5) groups. Adhesions and abscesses were noted at 7 d after CLP. RT-PCR assay was performed to assess the cecal tissue.

**RESULTS:** Adhesion formation was significantly reduced in PG0.25, 0.5, PL0.25, 0.5, and HA groups (2.5±0.7, 2.2±0.7, 3.8±1.0, 3.6±0.8, and 2.7±1.1, *P*<0.05). The incidence of abscesses was significantly reduced in all treated groups compared to RL group (58%, *P*<0.05). The urokinase-type plasminogen activator (uPA) gene expression was greatly up-regulated by increasing the concentration of polysaccharides. The urokinase-type plasminogen activator receptor (uPAR) and tumor necrosis factor (TNF)-α mRNA were highly expressed in PG0.25, 0.5, PL0.25, and 0.5 groups.

**CONCLUSION:** We concluded that 0.5% polysaccharide derived from PG and PL was the optimal concentration in preventing adhesion and abscess formation and may act by modulating activity of uPA and TNF-α in a rat peritonitis model.

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**Key words:** Adhesion; Abscess; *Phellinus gilvus*; *Phellinus linteus*, Polysaccharides

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**INTRODUCTION**

Intraperitoneal adhesions and abscesses are a major cause of morbidity and mortality in the adult intensive care unit. They are caused mainly by previous surgery and abdominal inflammation[1,2]. Abdominal infection is accompanied by peritoneal inflammation, including exudation of fibrinogen and fibrin formation into the abdominal cavity. In infectious conditions, these fibrin deposits may become a nidus for abscesses[3] and in turn become fibrous adhesions. Therefore peritoneal fibrinolytic system is crucial in preventing adhesion and abscess formation. Activation of fibrinolytic system results in the conversion of plasminogen into plasmin. The conversion is activated directly by tissue-type plasminogen activators (tPA) and uPA[4,5]. Various cells produce tPA, including endothelial cells, mesothelial cells, and macrophages. The uPA is also produced by the same cells and is equally effective in the degradation of fibrin[6].

Numerous agents have been investigated in the prevention of adhesion and abscess formation. Recently, it was reported that beta-glucan in polysaccharides from chemical was capable of reducing the frequency of adhesion by preventing with beta-glucanase[7]. It is a potent macrophage stimulator that enhances macrophage cytotoxicity and phagocytic capacity and induces production of TNF-α[8,9]. We have also previously demonstrated that 0.025% polysaccharides derived from PG and PL reduced both intraperitoneal adhesion and abscess formation by modulating activity of uPA and TNF-α produced from activated macrophages in a rat peritonitis model[10]. However, comparison of adhesion- and abscess-reducing capacity by varying concentrations of polysaccharides solutions derived from PG and PL has not been demonstrated to date.

Therefore, in this study, we hypothesize that intraperitoneal abscesses and adhesions could be reduced by increasing concentration of polysaccharides solutions derived from PG and PL, and differences of the effect could be related to uPA and TNF-α activity produced from activated macrophages. To test this hypothesis, we investigated differences by varying concentration of polysaccharides derived from PG and PL in preventing adhesion and abscess.
formation in order to investigate influence of the uPA and TNF-α gene expression in a rat peritonitis model.

MATERIALS AND METHODS
Preparation of materials
The fruiting body of PG was kindly provided by Gyeongbuk Agricultural Technology Administration (Daegu, Korea). A seed culture was grown in a 250-mL flask containing 50 mL of PMP medium (2.4% potato/dextrose broth plus 1% malt extract, 0.1% peptone) at 28 °C on a rotary incubator at 150 r/min for 4 d. To obtain fruiting bodies of PG, a culture was grown in oak sawdust block for 90 d. The yield of fruiting bodies was 97 g dried weight per block. PL used in this study was developed for 3 years in routine artificial mulberry cultures and purchased from Sanwhang Mushroom Co. (Andong, Korea). The fruiting body of PG and PL was cut into small pieces, dried at 40-50 °C for 48 h. It was homogenized, extracted by optimal water extraction conditions, distilled water (1:25) at 100 °C for 10 h, and concentrated at 80 °C in a rotary evaporator. The recovery procedure of the polysaccharides from the fruiting body of PG and PL followed an established method in our previous study[10]. The concentration of 0.025%, 0.25%, and 0.5% polysaccharides solutions was determined by measuring total sugar using the anthrone method[12] using glucose as the standard material. The 0.2% HA was prepared by adding distilled water at HA (HYAL®, Shinpoong Pharm. Co., Korea). It was filtered through a 0.22 μm membrane filter. All the materials were stored at 4 °C until used.

Animals
Ninety-six male Sprague-Dawley (SD) rats (Charles River Korea Inc., Korea) weighing 237 to 261 g were acclimated under the controlled conditions for 1 wk before the experiments. The animals were fed with commercial rat feed from Orient Inc., Korea. Food and water were provided ad libitum to the animals. The Guidelines for Animal Care and Use of Kyungpook National University approved the housing, care and use of animals, as well as procedures to minimize discomfort.

Surgical procedures
Bacterial peritonitis was induced by performing a CLP procedure according to Wichterman et al.[13]. Only water was provided in the 12 h preceding the experiments. The animals were weighed and anesthetized by intramuscular injection of a combination of ketamine (100 mg/kg) and xylazine (5 mg/kg). They breathed spontaneously throughout the procedures. The abdominal skin was disinfected with 70% alcohol. All procedures were performed under sterile conditions. Routine midline celiotomy was performed with a 3-cm incision and the peritoneum, the omentum, and between the bowel loops. The sum of these locations formed the total adhesion score (0-24).

Table 1 Grading of adhesions according to Zuhlke

| Grade | Description |
|-------|-------------|
| 0     | No adhesions |
| 1     | Filmy adhesions: gentle, blunt dissection required to free adhesions |
| 2     | Mild adhesions: aggressive blunt dissection required to free adhesions |
| 3     | Moderate adhesions: sharp dissection required to free adhesions |
| 4     | Severe adhesions: not dissectible without damaging organs |

Note: Locations scored included midline, adnexa/epididymal fat bodies, the upper abdomen (liver), the parietal peritoneum, the omentum, and between the bowel loops. The sum of these locations formed the total adhesion score (0-24).

Bacterial cultures
Samples of peritoneal fluid and abscesses were taken from all animals on the second postoperative day by swabs for verification of the induced peritonitis. The swabs were immediately introduced into medium and cultured semiquantitatively in aerobic and anaerobic conditions. Samples were incubated on blood and EMB agar for aerobic culture and layered on anaerobic blood agar and incubated in a Gas-Pak jar for anaerobic culture. After 24 and 48 h of incubation at 37 °C, growing colonies were identified with standard bacteriologic techniques.

Tissue collection
The adhesion-carrying cecal site was resected carefully. The

enrofloxacin (1 mg/kg) and 10 mL of isotonic sodium chloride solution subcutaneously for hydration. After 24 h, animals were weighed and the abdomen was reopened under the same anesthesia as the first celiotomy. Samples of peritoneal fluid were taken for microbiologic examination. The abdominal cavity was rinsed with 10 mL isotonic sodium chloride solution, and the cecum was resected. Before closure of the abdomen, the animals were randomly allocated to 8 groups of 12. One control group was treated with 8 mL of ringer lactate solution (RL group) and one other control group was treated with 8 mL of 0.2% HA solution (HA group) intraperitoneally through the urinary catheter. Six experimental groups were treated with 8 mL of 0.025%, 0.25%, and 0.5% polysaccharides derived from PG (PG0.025, 0.25, and 0.5 groups) and PL (PL0.025, 0.25, and 0.5 groups) intraperitoneally, respectively. All animals were given water only on the first postoperative day; standard rat chow and water ad libitum were provided on the second postoperative day. The animals were weighed again and killed with carbon dioxide asphyxiation a week after the first postoperative day. The abdomen was opened via a U-shaped incision for complete exploration. Adhesions and the incidence of abscesses were examined in a blind manner by one of us (HK Jin) according to the method of Zuhlke et al.[14], whereby grade 0 means no adhesions and grade IV means firm extensive adhesions that are dissectible only with sharp instruments, with organ damage almost unavoidable. Sites of adhesions scored included the midline, adnexa/epididymal fat bodies, the upper abdomen (liver), the parietal peritoneum, the omentum, and between the bowel loops. The total score of these six locations was noted as the total adhesion score (0-24) (Table 1).
Cecal tissue was cut longitudinally to remove food contents, washed with sterile phosphate-buffered saline (PBS). Half the animals in each group were fixed in 10% formalin in PBS for histopathologic evaluation and the remaining animals in each group were stored at 80°C for RT-PCR analysis until further processing.

**Histopathologic evaluation**

After routine tissue processing, serial sections (5 μm) were stained with hematoxylin and eosin (HE). The inflammatory reaction was assessed for each group by light microscopy. The grade of inflammation was assessed using a semi-quantitative scoring system, the inflammation grading scale. Grade 1 on this scale represents a mild inflammatory reaction with giant cells, occasionally scattered lymphocytes, and plasma cells. Grade 2 represents a moderate reaction with giant cells and increased admixed lymphocytes, plasma cells, eosinophils, and neutrophils. Grade 3 represents a severe inflammatory reaction with microabscesses present.

**RNA extraction**

Total cellular RNA was extracted from rat cecum using a monophasic solution of phenol and guanidine isothiocyanate (TRIzol Reagent, Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The purity and integrity of the RNA samples were assessed by A260/280 spectrophotometric measurements.

**RT**

A 1 μg portion of total RNA was subjected to first-strand cDNA synthesis in a 20 μL reaction mixture containing Moloney murine leukemia virus reverse transcriptase (10 U), dNTP mixture (2.5 mmol/L concentrations of each dNTP), oligo (dT)12-18 primers (10 μmol/L), and reaction buffer as supplied with the enzyme (50 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 10 mmol/L MgCl2, 0.5 mmol/L spermidine, and 10 mmol/L dithiothreitol). The samples were incubated in a TOUCHgene DNA thermal cycler (Techne (Cambridge) Limited, U.K.) at 42°C for 60 min followed by enzyme denaturation step at 94°C for 2 min. The reverse transcription mixture was stored at -80°C for use in PCR. All reagents were obtained from Promega (Madison, WI).

**PCR**

PCR was performed on 2 μL of reverse transcriptase product using Gene Tag (Nippon Gene Co., Ltd., Toyama, Japan) containing Tag DNA polymerase, dNTPs, buffer, and 0.5 μmol/L concentrations of each gene-specific forward and reward primers (obtained from Bioneer, Daejeon, Korea) in a total volume of 50 μL. Gene-specific oligonucleotide primers were designed from published rat sequences. Primers used for amplification: TNF-α: sense, 5'-TACTGAACTTGGGTGATGGTCC-3', antisense, 5'-CAGCTTGGTCTCCGGAAAGAAC-3'; uPA: sense, 5'-TGTGGATTTGGGCACAGATGC-3', antisense, 5'-GCTGTGGTCCCCCG-3'; glyceraldehydes-3-phosphate dehydrogenase (GAPDH): sense, 5'-TGAAGGTCGGGTGTGAACGGATTGGGC-3', antisense, 5'-CATGAGCCATGAGTTCCACACC-3'. The PCR was conducted in TOUCHgene DNA thermal cyclers. After an initial denaturation at 95°C for 5 min, amplification was conducted through 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C (GAPDH) or 60°C (for all the other transcripts) for 30 s and extension at 72°C for 45 s. Final extension was at 72°C for 10 min followed by a final hold at 4°C. Negative controls (PCR mixture without cDNA) and positive controls (PCR mixture with a standard cDNA sample) were included in preliminary PCR runs. Initial experiments were conducted to determine the optimal annealing temperature for each set of gene-specific primers (data not shown). The PCR products were separated by electrophoresis using 2% agarose gels stained with ethidium bromide to visualize cDNA products.

**Statistical analysis**

Values are expressed as mean±SD. Analysis of differences between treated groups and untreated groups was performed using analysis of variance followed by multiple comparisons and Fisher's LSD test using the SAS statistical package (release 8.1; SAS Institute Inc., Cary, NC). Differences at P<0.05 were considered statistically significant.

**RESULTS**

Following CLP, all rats showed symptoms of peritonitis-like apathetic behavior, ocular exudates, and piloerection. These symptoms resolved within 2 d following the celiotomy and removal of the necrotic, perforated cecum and peritoneal lavage. Survival rates in all groups were 100% by the end of the experiment.

**Body weights of the rats**

The mean±SD body weight of the rats was 248.1±12.3 g at the time of the first operation. The rats lost weight during peritonitis (240.2±12.8 g) and recovered weight by the end of the experiment (255.3±10.7 g) in RL, PG0.025, PL0.025, and HA groups. The differences in weight gain at the time of the first operation. The rats lost weight during peritonitis (240.2±12.8 g) and recovered weight by the end of the experiment (255.3±10.7 g) in RL, PG0.025, PL0.025, and HA groups. The differences in weight gain were not statistically significant among the groups. But, the weight gain in the PG0.025, 0.5, PL 0.25, and 0.5 groups (270.2±12.8 g) was higher than that in RL, PG0.025, PL0.025, and HA groups.

**Microbiological examination**

Culture results of the peritoneal fluid taken at the day of cecal resection revealed polymicrobial intraperitoneal infection. The most frequently isolated microorganisms were *Escherichia coli* (50.9%), *Proteus* species (57.5%), *Staphylococcus* (48.1%), *Streptococcus* (25%), Gram-positive *Bacillus* species (15%), and *Klebsiella* (4%). *E. coli* (84.7%) was the organism isolated most frequently from abdominal abscesses.

**Total adhesion score and site of adhesion**

The PG polysaccharide solutions Rats treated with 0.25% and 0.5% of PG polysaccharide solutions had a significantly lower total adhesion score compared to that of the RL group (P<0.05). The total adhesion score of rats treated
with the RL solution was 12.3±5.2. Group treated with 0.25% (2.5±0.7) and 0.5% (2.4±0.7) of PG polysaccharide solutions was lower than the group treated with 0.025% (5.8±1.0) solution in total adhesion score (*P<0.05) (Figure 1). There was no significant difference in total adhesion score between PG0.25 and PG0.5 group. The prevention effect of adhesion of PG0.25 and 0.5 groups was slightly higher than that in PL0.25, PL0.5, and HA (2.7±1.1) groups. There was no statistical difference in total adhesion score between PG0.25 and 0.5% treated rats. The site of the adhesions did not differ among the PG groups. Most of the adhesions were found between the bowel loops (81.1%), adnexa/epididymal fat bodies (62.5%), and the omentum (43.2%) in the PG groups (*P<0.05) (Figure 2).

**The PL polysaccharide solution** Rat treated with 0.25 and 0.5% of PL polysaccharide solutions had a significantly lower total adhesion score compared to RL group (*P<0.05) (Figure 1). The prevention effect of adhesion was slightly lower than the effect of PG0.25, PG0.5, and HA groups. Group treated with 0.25% (3.8±1.0) and 0.5% (3.6±0.8) of PL polysaccharide solutions was lower than the group treated with 0.025% PL (5.6±1.8) solution in total adhesion score (*P<0.05). There was no significant difference in total adhesion score between PL0.25 and PL0.5 groups like groups treated with 0.25% and 0.5% of PG polysaccharide solutions. The prevention effect of adhesion of PL0.25 and 0.5 groups was slightly lower than that in HA (2.7±1.1) groups. One of 12 (17%) PL-treated rats had grade IV adhesions. The site of the adhesions did not differ among the PL groups. Site of the adhesions was similar to the site of rats treated PG (Figure 2).

**Abscesses**

The incidence of intraperitoneal abscess significantly reduced in all treated groups (*P<0.05) compared to that in RL group. No abscess occurred in rats treated with PG 0.25 and 0.5. Rats treated with HA (3 of 12, 25%) reduced the incidence of abscesses compared to RL (7 of 12, 58%).

**Histologic evaluation**

Mostly, the inflammatory reaction is dominant at mesenteric fat and serosal surface of cecum. Rats treated with PG0.25 showed markedly reduced inflammatory reaction compared to RL (Figure 3). The RL group showed increased admixed lymphocytes, plasma cells, eosinophils, and neutrophils (grade 3 on the inflammation grading scale). The grade of

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**Figure 1** Total adhesion score of intraperitoneal adhesion in each group (in all rats). The animals were killed one week after the first postoperative day. The abdomen was opened for complete exploration. Rats treated with 0.25 or 0.5% polysaccharides solutions from both PG and PL showed a significantly lower total adhesion score compared with RL group (*P<0.05 vs RL group).

**Figure 2** The site of the intraperitoneal adhesions (in all rats). After the sacrifice, sites of adhesions were observed in the midline, adnexa/epididymal fat bodies, the upper abdomen (liver), the parietal peritoneum, the omentum, and between the bowel loops. Most of the adhesions were found between the bowel loops, adnexa/epididymal fat bodies, and the omentum compared with other sites (*P<0.05).
inflammatory response for the PG0.5 and PL0.5 groups (1.1±0.4 and 1.2±0.4) was significantly lower than the grade for the RL (2.9±0.4) ($P<0.05$) and slightly lower than PG0.025 and 0.25 (1.7±0.5 and 1.3±0.5), PL0.025 and 0.25 (1.8±0.4 and 1.5±0.5), and HA group (1.6±0.5) (Figure 4).

**DISCUSSION**

Abdominal infections are associated with fibrin deposit, which may cause clinically significant adhesion and abscess
formation. Adhesions are the most common cause of intestinal obstruction in developed countries and important cause of chronic abdominal and pelvic pain[6-10].

The CLP model has been used frequently for elucidating the pathophysiology of abdominal inflammation and developing new treatment modalities[24], because it is caused by the intraperitoneal adhesions that are promoted by surgical trauma, bacterial contamination, allergic reactions to foreign bodies left in the abdomen, and tissue ischemia. Thus, in our study, we used the CLP model that underwent cecal resection to induce adhesion and abscess formation in infectious environment.

The number of different agents has been studied in the search to prevent intraperitoneal adhesions, with variable results. Many studies showed HA decreases inflammation[21], interferes with fibrin formation[22], prevents adhesion and abscess formation[23], and stimulates fibrinolytic and TNF-α response[24,25]. In our study, we treated with 0.2% HA to prevent intraperitoneal adhesion in rats and this solution significantly reduced adhesion and abscess formation by modulating uPA and TNF-α production. This is in accordance with the results from earlier studies[23-25] for the prevention of intraperitoneal adhesions formation in rats using 0.2% HA. And, we have previously demonstrated that 0.025% polysaccharides derived from PG and PL reduced both intraperitoneal adhesion and abscess formation by modulating activity of uPA and TNF-α produced from activated macrophages in a rat peritonitis model[26]. In the present study, we treated with varying concentrations of 0.025%, 0.25%, and 0.5% of PG and PL in rats. It resulted that 0.25 and 0.5% solutions had higher adhesion- and abscess-reducing capacity than 0.025% solution in rats. The aP/A gene expression was greatly up-regulated by increasing concentration of the solutions. Therefore, we think that PG and PL may act in a dose-dependent manner in the prevention of adhesion and abscess formation and the action may be determined by modulating fibrinolytic capacity of uPA produced from macrophages.

In short, PG and PL used in our study are fungi belonging to the species of Hymenochaetaceae (Basidiomycetes) and found mainly in tropical areas of America and Africa[24]. Polysaccharides isolated from them have received special attention due to their potent pharmacological activities. PL is well known as one of the most popular medicinal mushrooms due to its high anti-tumor[27] and immunostimulating activities[28]. It has been used medicinally in Korea and Japan. Recently, it was reported that polysaccharide solutions from PL and PG had anti-inflammatory activity related to arthritis, septic shock, and pulmonary inflammation[29,30]. Thus, we think that these anti-inflammatory activities of natural products like PG and PL may be beneficial in the treatment of intraperitoneal adhesion related to inflammation.

PG has other advantages over PL in that it has a very short growth period (3 mo) compared to PL (2-3 years) making it cheaper to produce and the safety of acute single orally-administered dose of PG has been demonstrated. In the present study, although it is not a statistically significant analysis, polysaccharides from PG had better capacity than PL in the prevention of adhesion and abscess formation. Therefore, this suggests an additional potential therapeutic role for PG in the treatment of inflammation in future.

In an experimental study, Bedirli et al[1] showed that beta-glucan in polysaccharides had a positive weight gain effect on the animals. In our study, although it is not a statistically significant analysis, weight gain in the 0.25% and 0.5% of PG and PL groups is higher than that in RL, HA, PG0.025, and PL0.025 groups. That is to say, it showed a positive weight gain in groups treated with high dose, 0.25% and 0.5% of polysaccharides. However, the precise mechanism of weight gain in these animals is uncertain. We postulate that this could be related to a difference in the mechanism by which adhesion formation is reduced.

Many studies showed that beta-glucan in polysaccharides is a potent stimulator of macrophage functions and it induces TNF-α production in wound tissue[8,9]. But the role of TNF-α in adhesion formation is not clear. More recently, Reijnen et al[23] reported that HA counteracts the fibrinolytic decline induced by TNF- α. Boyce et al[25] indicated that TNF-α down-regulates fibroblastic collagen synthesis within experimental wounds and HA stimulates TNF-α production by human macrophages. In our study, TNF-α mRNA was highly expressed in the PG0.25, 0.5, PL_0.25, and 0.5 groups compared to the RL group. This is in accordance with the results from Abel et al[21], which indicated that beta-glucan induced TNF-α production in wound tissue. In HA group, the level was slightly expressed compared to that of RL group. This is also in accordance with the results from Reijnen et al[24] and Boyce et al[25]. We infer that 0.25% and 0.5% of polysaccharides solutions derived from PG and PL stimulate macrophage activity and increase secretion of uPA, uPAR, and TNF-α by the stimulated macrophage activity. Thus, we conclude that high dose of polysaccharides solutions derived from PG and PL decrease adhesion formation by increasing macrophage activity and enhancing fibrinolytic activity.

In summary, high dose of polysaccharides solutions derived from the fungi, PG and PL are pharmacologic agents that rapidly enhance host resistance to a variety of biological insults through the fibrinolytic system and this involves macrophage activation. In the present study, 0.25 and 0.5% of polysaccharides significantly decreased intraperitoneal adhesion and abscess formation in a rat peritonitis model. It was as effective as HA in the prevention of intraperitoneal adhesion and abscess formation. Additional studies will help elucidate whether the use of such polysaccharides and HA in combination may have wider clinical application.

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