The Effect of Polysaccharides and Carboxymethylcellulose Combination to Prevent Intraperitoneal Adhesion and Abscess Formation in a Rat Peritonitis Model

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ABSTRACT. Polysaccharides isolated from fungi, Phellinus spp. is well-known material with anti-tumor and anti-inflammatory properties. We have assessed the adhesion- and abscess-reducing capacity of carboxymethylcellulose (CMC) and polysaccharides from Phellinus spp. combination in a rat peritonitis model. In 72 Sprague-Dawley rats, experimental peritonitis was induced by means of the cecal ligation and puncture model (CLP). After 24 hr, the abdomen was reopened and the ligated cecum was resected. Peritoneal fluid samples were taken for microbiological examination. Rats were randomly assigned to 6 groups: ringer lactate solution (RL group), polysaccharides from Phellinus gilvus (PG group) and Phellinus linteus (PL group), carboxymethylcellulose (CMC group), and their combinations (PG+CMC and PL+CMC groups). Adhesions and abscesses were noted at day 7 after CLP. RT-PCR assay for urokinase-type plasminogen activator (uPA), its cellular receptor (uPAR), and tumor necrosis factor (TNF)-α was performed to assess the cecal tissue. Microbiological examination showed polymicrobial bacterial peritonitis. Adhesion formation was significantly reduced in PG+CMC and PL+CMC groups (P<0.05). The incidence of abscesses was reduced in all treated groups except the RL group (P<0.05). uPA, uPAR, and TNF-α mRNA were highly expressed in the PG+CMC and PL+CMC groups, as compared to the RL group. We concluded that the combination of polysaccharides and CMC had significant adhesion- and abscess-reducing effects compared with their single treatment and the effects may act by modifying the fibrinolytic capacity of uPA, uPAR and TNF-α produced from activated macrophages in a rat peritonitis model.

KEY WORDS: adhesion, peritonitis, Phellinus spp., polysaccharides, rat.

Peritoneal injury from a variety of causes leads to intraperitoneal inflammation and it is accompanied by fibrin deposition in the abdominal cavity. These fibrin deposits may in turn become fibrous adhesions due to collagen deposition by invading fibroblasts. In infectious conditions, adhesions may form as a part of an abscess. Both intraperitoneal adhesions and abscesses cause significant clinical morbidity and mortality [6, 20, 25]. Therefore degradation of fibrin is a significant target in preventing adhesion and abscess formation. Activation of the fibrinolytic system can result in lysis of fibrin deposits. If this mechanism fails, the adhesions will become fibrous [12]. In shortly, this system converts plasminogen into plasmin and the conversion is activated directly by tissue-type plasminogen activator (tPA) and uPA [13, 24]. Various cells produce tPA, including endothelial cells, mesothelial cells, and macrophages. uPA is produced by the same cells and is equally effective in the degradation of fibrin [21].

Numerous agents have been investigated for the prevention of adhesions and abscess formation. Polysaccharides isolated from mushrooms such as Phellinus gilvus (PG) and Phellinus linteus (PL) used in our study are well-known medicinal sources due to their anti-tumor [11], immunostimulating [19], and anti-inflammatory activities [15, 17, 18]. Their anti-inflammatory activities suggest that these natural products may be beneficial in the treatment of intraperitoneal adhesion related to inflammation. In short, PG and PL are mushrooms belonging to the Hymenochaetaceae basidiomycetes. There are approximately 220 known species of Phellinus and they are found mainly in tropical areas of America and Africa [5].

The capacity of CMC solution to prevent the formation of postsurgical adhesions in an infectious or noninfectious environment is well documented [7–9, 22, 23]. The studies showed that intraperitoneal treatment with 8 ml of 1.7% CMC solution reduced both adhesion and abscess formation in a rat peritonitis model. Furthermore, we had found that polysaccharides isolated from the mushrooms (PG and PL) reduced both adhesion and abscess formation through preliminary experiments. Therefore, in this study, we hypothesize that intraperitoneal abscesses and adhesions could be reduced in combinations of polysaccharides and CMC groups due to their synergic effect and it could be related to uPA, uPAR and TNF-α activity produced by activated macrophages. To test this hypothesis, we investigated the prevention of adhesion and abscess formation by a combination of CMC and polysaccharides compared to their single CMC or polysaccharides treatment, in order to investigate the influence of uPA, uPAR 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...
Fig. 1. Schematic diagram depicting the separation of polysaccharides from extracts derived from the fruiting body of PG and PL.

The animals were weighed and anesthetized by intramuscular injection of a combination of 100 mg/kg of ketamine hydrochloride (Ketamine®, Yuhan Co., Korea) and 5 mg/kg of xylazine hydrochloride (Rompun®, Bayer Korea Ltd., Korea). All procedures were performed under sterile conditions. Routine midline celiotomy was performed with a 3 cm incision and the cecum was exposed. The cecum was ligated just distally to the ileocecal valve with a 3–0 polyglactin 910 (VICRYL®, ETHICON, INC., Johnson & Johnon Co.) suture to avoid intestinal obstruction, punctured once with a 19-G needle, squeezed gently to force out a small amount of feces, and then returned to the abdominal cavity. After closing the abdomen in 2 layers, the animals received 1 mg/kg of enrofloxacin (Baytril®, Bayer Korea Ltd., Korea) and 10 ml of isotonic sodium chloride solution subcutaneously for analgesia and hydration. After 24 hr, the animals were weighed and the abdomen was reopened under the same anesthesia as in the first celiotomy. Samples of peritoneal fluid were taken for microbiologic examination. The abdominal cavity was rinsed with 10 ml of isotonic sodium chloride solution, and the cecum was resected. Before closure of the abdomen, the animals were randomly allocated to 6 groups of 12. One control group was treated intraperitoneally with 8 ml of ringer lactate solution (RL group). Five groups were treated intraperitoneally with 8 ml of 0.025% polysaccharides isolated from PG (PG group) and PL (PL group), 0.2% CMC (CMC group), and their combinations (PG+CMC and PL+CMC groups) through a urinary catheter. 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 sacrificed with carbon dioxide asphyxiation one 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 blinded manner by an irrelevant person according to the method of Zuhlke et al. [27], whereby grade 0 means no adhesions and grade IV means firm extensive adhesions that are dissectable 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 for these six locations was noted as the total adhesion score (0–24) (Table 1).

**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 [23].

**Tissue collection:** The adhesion-carrying cecal site was resected carefully, the cecal tissue cut longitudinally to remove food contents, and washed with sterile phosphate-buffered saline (PBS). Half of the tissues in each group were fixed in 10% formalin in PBS for histopathologic evaluation and the other half of the tissues in each group were
stored at –80°C for RT-PCR analysis until further processing.

RNA extraction: Total cellular RNA was extracted from rat cecum with a monophasic solution of phenol and guanidine isothiocyanate (TRIsol Reagent, Gibco) according to the manufacturer’s instructions. The purity and integrity of the RNA samples were assessed by OD 260/280 spectrophotometric measurements.

Reverse transcription: 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 mM of each dNTP), oligo(dT)12-18 primers (10 µM), and reaction buffer as supplied with the enzyme (50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl2, 0.5 mM spermidine, and 10 mM dithiothreitol). The samples were incubated in a TOUCHgene DNA thermal cycler (Techne (Cambridge) Limited, U.K.) at 42°C for 60 min followed by an 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 Corp. (Madison, WI, U.S.A.).

PCR: PCR was performed on 2 µl of reverse transcriptase product with Gene Taq (Nippon Gene Co., Ltd., Toyama, Japan) containing Taq DNA polymerase, dNTPs, buffer, and 0.5 µM of each gene-specific forward and reverse primers (obtained from Bioneer Corp., 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'-TACTGAACT-TCCGGGTGTATTGGTCC-3', antisense, 5'-CAGCCT-TGTCCTTTGAAGAGAACC-3'; uPA: sense, 5'-TCGTAATCAGCCTAAGAAGAGTACG-3', antisense, 5'-TTACAACGACATTTTCAGGTCC-3'; uPAR: sense, 5'-CAGAACACTGTATTGAAAGTGTTGACGCC-3', antisense, 5'-TCCAAGCACGTATCTCAGCTCC-3'; antitense, 5'-TCCAAGCACGTATCTCAGCTCC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense, 5'-TGAGTTCCGTT-GAACGGATTGGC-3', antisense, 5'-CATGTAGGC-TCGGGGTGATTGGTCC-3'. The PCR products were separated by electrophoresis with 2% agarose gels stained with ethidium bromide to visualize cDNA products.

Histopathologic evaluation: The cecal tissues were fixed in 10% formalin in PBS for at least 1 hr. After routine tissue processing, serial sections (5 µm) were stained with hematoxylin and eosin (H&E). The inflammatory reaction was assessed for each group by light microscopy. The grade of inflammation was assessed with a semiquantitative scoring system on the inflammation grading scale [14]. Grade 1 on this scale represents a mild inflammatory reaction with giant cells, occasional 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.

Table 1. Grading of adhesions according to Zuhlke et al.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
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<tbody>
<tr>
<td>0</td>
<td>No adhesions</td>
</tr>
<tr>
<td>I</td>
<td>Filmy adhesions: gentle, blunt dissection required to free adhesions</td>
</tr>
<tr>
<td>II</td>
<td>Mild adhesions: aggressive blunt dissection required to free adhesions</td>
</tr>
<tr>
<td>III</td>
<td>Moderate adhesions: sharp dissection required to free adhesions</td>
</tr>
<tr>
<td>IV</td>
<td>Severe adhesions: not dissectible without damaging organs</td>
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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).

RESULTS

After CLP, all rats had symptoms of intra-peritoneal sepsis. They demonstrated apathetic behavior, diarrhea, ocular exudates and piloerection. These symptoms resolved within 2 days after the receliotomy and removal of the necrotic, perforated cecum and peritoneal lavage. Survival rates in all groups were 100% at the end of the experiment. Weight loss was observed in the first week. After 1 week, the rats regained weight. The mean ± SD body weight of the rats was 261.9 ± 20.3 g at the time of the first operation. The rats lost weight during peritonitis (247.2 ± 20.3 g) and recovered weight by the end of the experiment (255.3 ± 10.7 g) in all groups. The differences in weight loss or weight gain were not statistically significant among the groups.

Bacterial cultures: Bacterial culture results for the peritoneal fluid taken at the day of cecal resection revealed mixed aerobic and anaerobic microorganisms. The most frequently isolated microorganisms were Escherichia coli (67.8%), Proteus species (55.1%), Staphylococcus (43%), Streptococcus (18.4%), Gram-positive Bacillus species (7.9%) and Klebsiella (2.3%). Escherichia coli (88.1%) was the organism isolated most frequently from abdominal abscesses.

Total adhesion score and site of adhesion: Rats treated with a combination of polysaccharides and CMC (PG or PL
+ CMC groups) had a significantly lower total adhesion score than that of their single (PG, PL, and CMC groups) and RL treatment groups (RL group) \( P<0.05 \). There was no significant difference in the total adhesion score between PG+CMC (2.5 ± 0.9) and PL+CMC (3.3 ± 0.8) groups. The total adhesion score for rats treated with the RL solution was 10.5 ± 4.3. Single groups treated with PG (5.6 ± 1.0), PL (5.3 ± 1.3) and CMC (6.1 ± 2.2) were lower than the RL group in the total adhesion score \( P<0.05 \) (Fig. 2). There was no statistical difference in the total adhesion score among the single treatment groups. Nine of 12 (75%) RL treated rats had grade IV adhesions, in contrast to only 1 of 12 (8.3%) PG+CMC and 2 of 12 (16.6%) PL+CMC treated rats. The site of the adhesions did not differ among all groups. Most of the adhesions were found between the bowel loops (75.8%), adnexa/epididymal fat bodies (67.6%) and the omentum (52.7%) in all groups \( P<0.05 \) (Fig. 3).

**Abscesses:** The incidence of intraperitoneal abscesses was significantly reduced in all treated groups \( P<0.05 \) compared to that in the RL group. No abscess occurred in rats treated with a combination of polysaccharides and CMC (PG or PL+CMC groups). Rats single treated with PG (3 of 12, 25%), PL (2 of 12, 16.7%) and CMC (3 of 12, 25%) had a reduced incidence of abscesses compared to RL (8 of 12, 66.7%).

**TNF-α, uPA, and uPAR expression:** Experiments were carried out to demonstrate the effect of single and combination groups on the gene transcription of TNF-α, uPA, and uPAR. In the combination of polysaccharides and CMC groups (PG or PL+CMC), TNF-α mRNA was highly expressed, as compared to the RL group. The level was slightly increased compared to RL in single treatment groups (PG, PL and CMC groups). The uPA gene expression was greatly increased in the combination and CMC groups, compared to the RL group. In the single treatment PG and PL groups, the level was only slightly expressed compared to the RL group. The uPAR mRNA was expressed at the highest levels by the treatment with a combination of polysaccharides and CMC. In single treatment groups, the level was only slightly increased compared to the RL group. The GAPDH transcript levels in all groups were the same (Fig. 4).

**Histologic evaluation:** The inflammatory reaction is the most dominant at the mesenteric fat and serosal surface of the cecum. Rats treated with a combination of PG+CMC and PL+CMC demonstrated a noticeably reduced inflammatory reaction compared to rats treated with RL. The RL group showed increased admixed lymphocytes, plasma cells, eosinophils and neutrophils (grade 3 on the inflammation grading scale) (Fig. 5). The grade of inflammatory response for the PG+CMC (1.2 ± 0.4) and PL+CMC groups (1.7 ± 0.5) was significantly lower than grade for the RL group (2.7 ± 0.5) \( P<0.05 \) (Fig. 6). The grades of the PG, PL and CMC groups were 2.0 ± 0.6, 2.2 ± 0.8 and 2.2 ± 0.8, respectively. There was no statistical difference in the grade of inflammation among the single treatment groups.

**DISCUSSION**

Numerous agents and methods have been tested to prevent postoperative intraperitoneal adhesion and abscess formation in animal and clinical studies; most of them are not successful [3, 10], so that their clinical use is somewhat limited. Our present study demonstrated that intraperitoneal treatment with a combination of 0.025% polysaccharides (from PG and PL) and 0.2% CMC solution reduced intraperitoneal adhesion and abscess formation in a rat peritonitis model.

The fruiting bodies of PG and PL are very expensive due to having various biological effects such as anti-tumor [11], immunostimulating [19], and anti-inflammatory [15, 17, 18] activities in other countries as well as Korea. Thus, the combination of a low concentration of polysaccharides isolated from them and anti-adhesive agents (eg, CMC and hyaluronic acid) have many advantages in medical cost-cutting as well as health benefits.

The CMC used this study is an absorbable hemostatic agent that is formed by dissolving pure \( \alpha \)-cellulose in an alkaline solvent. The adhesion-reducing capacity of this solution is well established in animals [8, 9, 22]. Reijnen et al. [23] reported that intraperitoneal treatment with 8 ml of 1.7% CMC solution significantly reduced both adhesion and
abscess formation in a rat peritonitis model. In the present study, we found that the lower concentration of 0.2% CMC also had an adhesion- and abscess-reducing capacity. On the other hand, intraperitoneal treatment with a low concentration combination of polysaccharides and CMC had significant adhesion- and abscess-reducing effects compared with a single 0.2% CMC or 0.025% polysaccharide treatment. Therefore, we judged that the combination of lower concentrations of polysaccharides and CMC had an effect similar to that of a single use of 1.7% CMC in preventing adhesion and abscess formation, but the mechanism of CMC is not yet known precisely. In our study, the uPA and uPAR were expressed at high levels on treatment with CMC. We therefore suggest that this solution might reduce adhesion and abscess formation by modifying uPA and uPAR production.

Many studies have shown that polysaccharides from various products are potent stimulators of macrophage functions and they induce TNF-α production in wound tissue [1, 4], but the role of TNF-α in adhesion formation is not clear. But, in our study, TNF-α mRNA was highly expressed in combination polysaccharide and CMC groups. The level was also slightly increased in single treatment groups, so that we can conclude that polysaccharides (from PG and PL)
Fig. 6. The grade of inflammatory reaction in each group (n=12). * P<0.05 compared to RL group.

and CMC decreased adhesion formation by increasing macrophage activity and enhancing fibrinolytic activity.

In conclusion, a combination of polysaccharides and CMC is better than single treatment in preventing intraperitoneal adhesion and abscess formation in a rat peritonitis model. We suggest that it is due to their synergic effect related to uPA, uPAR and TNF-α activity produced from activated macrophages. Additional studies will help elicit whether the use of combinations of such polysaccharides and other anti-adhesive agents may have wider clinical applications.

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