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Oral administration of heparin or heparosan increases the *Lactobacillus* population in gut microbiota of rats

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ABSTRACT

Heparin and heparosan have been confirmed to be effective blockers in inhibiting adhesion of pathogens *in vitro*. However, their effects on gut microbiota *in vivo* remain unknown. Here we have studied the effects of oral administration of heparin or heparosan on gut microbiota in rats by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Results showed that the predominant bacterial communities in the feces of heparin- or heparosan-treated animals were different from those of the saline-treated animals, with increased *Lactobacillus* spp. and decreased *Enterococcus* sp. Different DGGE banding patterns were also observed for the subpopulations of *Lactobacillus* and *Bacteroides* groups. In conclusion, heparin or heparosan may be used as an effective gut microbiota modulator by increasing the subpopulation of *Lactobacillus*.

1. Introduction

The human endogenous gut microbiota is essential for the health (Eckburg et al., 2005). Aberrant gut microbiota has been shown to be associated with some intestinal disorder or diseases, such as irritable bowel syndrome (IBS) and ulcerative colitis (UC). In IBS patients, both increase and decrease of variation of microbiota diversity have been reported (Codling, O'Mahony, Shanahan, Quigley, & Marchesi, 2010; Salonen, de Vos, & Palva, 2010). Noor et al. (2010) found that the presence of some *Bacteroides* spp. and *Parabacteroides* sp. in healthy volunteers distinguished them from IBS and UC patients.

Due to the complex nature of gut bacterial communities, analysis of microbiota is often conducted with molecular techniques instead of cultivation techniques. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) (Muyzer, de Waal, & Uitterlinden, 1993) is based on the separation of PCR-amplified fragments of genes coding for conserved 16S rRNA from a mixed sample and is able to identify the constituents which represent only 1% of the total population. The banding patterns of PCR amplicons generated by DGGE can be compared to evaluate the relative similarity of microbial communities from different treatments.

In our previous study, heparin and heparosan (the biosynthetic precursor of heparin or heparan sulfate) showed selective anti-adhesion abilities to pathogenic and probiotic strains (Chen, Ling, Duan, & Zhang, 2012). Both heparosan and heparin blocked the adhesion of *Escherichia coli*, *Pasteurella multocida*, and *Staphylococcus aureus*, but they did not block the adhesion of *Lactobacillus rhamnosus* to enterocytes and mucus *in vitro* (Chen et al., 2012). The adhesion targets of many microorganisms have been identified as heparan sulfate on mammalian cells. Exogenous heparin acting as receptor mimicry, could block the bacterial exploitation of host heparan sulfate and inhibit the adhesion and dissemination of pathogens in the host (Arciola et al., 2003; Fallgren, Andersson, & Ljungh, 2001; Fears & Woods, 2006; Frick, Schmidtchen, & Sjobring, 2003; Gu, Wang, Guo, & Zen, 2008; Henry-Stanley, Hess, Erickson, Garni, & Wells, 2003; Henry-Stanley, Hess, Erlandsen, & Wells, 2005; Hess, Henry-Stanley, Erlandsen, & Wells, 2006; Menozzi et al., 2002; Rabenstein, 2002).

The selective anti-adhesion abilities of heparin and heparosan indicated that they might affect the components of gut microbiota differently and thus modify the gut microbiota *in vivo*. There were some trials of intravenous administration of heparin to treat UC (Head & Jurenka, 2003). We think the effectiveness of heparin may be associated with the modification of gut microbiota. However, none of previous studies determined the changes of gut microbiota

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Table 1
Primers used in this study.

Primer	Sequence (5′–3′)	Reference
P2	5′-ATTACCGCGGCTGCTGG-3′	Muyzer, de Waal, and Uitterlinden, 1993
P3	5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′	
LacI	5′-AGCAGTAGGGAATCTTCCA-3′	Walter, Hertel, Tannock, Lis, Munro, and Hammes, 2001
Lac2-GC	5′-CGCCCGGGGCGCGCCCCGGGCGGCCCGGGGGCACCGGGGGATTYCACCGCTACACATG-3′	
Bfr-F	5′-CTGAACCAGCCAAGTAGCG-3′	Liu, Song, McTeague, Vu, Wexler, and Finegold, 2003
Bfr-GC-R	5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCGCAAACTTTCACAACTGACTTA-3′	
Clept-F	5′-GCACAAGCAGTGGAGT-3′	Shen et al., 2006
Clept-GC-R3	5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCTTCCTCCGTTTTGTCAA-3′	

under the treatment of heparin. While intravenous administration of heparin against UC had a high risk of bleeding (Head & Jurenka, 2003), it remains unknown whether oral-administered heparin is effective on the gut microbiota. In the present study, we investigated the impact of oral administration of heparin on the gut microbiota using PCR-DGGE analysis of bacterial communities in fecal samples from rats. We also aimed to understand the acting mechanisms of heparin by comparing the sulfated heparin with nonsulfated heparosan.

2. Materials and methods

2.1. GAGs

Heparin (unfractioned) was purchased from Hebei Changshan Biochemical Pharmaceutical Co., Ltd. (Shijiazhuang, Hebei, China). The heparosan was prepared from fermentation broth of type D P-934 *P. multocida* (*P. multocida* subsp. *multocida* ATCC® 12948™) in brain–heart infusion (BHI) broth with a modified method (DeAngelis & Padgett-McCue, 2000; Chen et al., 2012).

2.2. Animals and treatments

Sprague-Dawley rats were purchased from Laboratory Animal Center of Shandong University (Jinan, Shandong, China). Forty Sprague-Dawley rats (20 male and 20 female, 75–95 g) were housed under controlled humidity (40–60%) and temperature (20–24 °C) with a 12 h light–dark cycle according to China GB 14295-2001 (Laboratory animal-requirements of environment and housing facilities). The animals were acclimated to the laboratory for two days and then randomized into five groups, *i.e.* the natural saline group, the high dose heparin group (10 mg/kg), the low dose heparin group (5 mg/kg), the high dose heparosan group (10 mg/kg) and the low dose heparosan group (5 mg/kg). Each group was composed of four male and four female rats and all animals had free access to food and water. All animals were orally administrated with aforementioned various doses of heparin or heparosan on a daily basis for two weeks. Fresh fecal samples were collected before the treatment, and on the 14th days of the treatment. The fecal samples were stored at −80 °C before preparation of total DNA.

2.3. Total bacterial DNA preparation

To extract fecal microbial cells, 1 g of feces was suspended in 35 mL of anaerobic phosphate-buffered saline (PBS, 0.1 M, pH 7.0) following steps below. A volume of 15 mL of PBS was added and homogenized by vortex for 10 min at high speed. Then another 10 mL of PBS was added and the mixture underwent further vortex for 3–5 min. The last 10 mL of PBS was added and mixed thoroughly. The suspension was centrifuged at 200 × *g* for 5 min and the supernatant was transferred into a new tube. After repeating the previous step twice, the supernatant was centrifuged at 9000 × *g* for 5 min. The pellet was washed twice with anaerobic PBS. Finally, the pellet was re-suspended in 10 mL of PBS and aliquoted into Eppendorf tubes and stored at −80 °C. Total bacterial DNA was prepared with a slightly modified method of protease K-SDS and freezing-thawing followed by chloroform/isoamyl alcohol extraction (Zijnge et al., 2006).

2.4. PCR amplification

All primers used in this study are listed in Table 1. The PCR amplification of the V3 region of the 16S rRNA gene was carried out with primers P2 and P3 according to the protocol described by Muyzer et al. (1993) in a thermocycler PCR system (MJ Mini™, Bio-Rad, USA). The 25 μL of PCR reaction mixture contained 0.5 μM of each primer, 2 mM $MgCl_2$, 0.625 U *Taq* polymerase (Fermentas, China) and 20 ng of the total fecal DNA. After the initial amplification, a reconditioning PCR method was performed to decrease heteroduplexes formation (Thompson, Marcelino, & Polz, 2002). PCR amplifications of *Lactobacillus*, *Bacteroides*, and *Clostridium* were carried out with the protocols described by Walter et al. (2001), Liu et al. (2003), and Shen et al. (2006), respectively.

2.5. DGGE

Amplicons of the V3 region of the 16S rRNA were separated by DGGE using a Dcode System apparatus (Bio-Rad, USA) in an 8% (w/v) acrylamide gel with a gradient 26.5–52%. The gel was electrophoresed at the constant voltage of 200 V and a temperature of 60 °C for 180 min. The denaturing gradients ranging 35–55% were used for the separation of amplicons of *Lactobacillus* and *Clostridium*, whereas gradients 22.5–45% were used for *Bacteroides*. After electrophoresis, the gels were stained with ethidium bromide and visualized on a GelDoc-It Imaging System (UVP, USA).

2.6. Statistical analysis

The DGGE banding patterns from three replicates in each group were digitalized by Quantity One software (Bio-Rad, USA). Each DGGE band was defined as one operational taxonomic unit (OTU) or phylotype. The intensity and relative position of each band were determined manually with background subtraction. The intensity of each band was expressed as percentage of the integrated intensity of the entire lane. The matrix of intensity and relative position was analyzed by principle component analysis (PCA) using SPSS software.

2.7. Sequence analysis of DGGE bands

Important DGGE bands were excised from the gel and incubated in 50 μL of sterile distilled water at 4 °C overnight. PCR amplifications of the DNA fragments from the excised gel were carried out according to the same protocols described above with the