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Quantification of Propionic acid in the bovine spinal disk after infection of the tissue with *P. acnes* bacteria

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Abstract

Study Design—Basic Research

Objective—The goal of this study was to investigate whether *P. acnes* infection of the intervertebral disc can be detected noninvasively by NMR spectroscopy.

Summary of Background Data—Microbiological studies of surgical samples suggest that a significant subpopulation of back pain patients may have occult disc infection with *P. acnes* bacteria. This hypothesis is further supported by a double-blind clinical trial showing that back pain patients with Modic type 1 changes may respond to antibiotic treatment. Because significant side effects are associated with antibiotic treatment, there is a need for a noninvasive method to detect whether specific discs in back pain patients are infected with *P. acnes* bacteria.

Methods—*P. acnes* bacteria were obtained from human patients. NMR detection of a propionic acid in the bacteria extracts was conducted on 500MHz high-resolution spectrometer while *in vivo* NMR spectroscopy of an isolated bovine disk tissue infected with *P. acnes* was conducted on 7T MRI scanner.

Results—NMR spectra of *P. acnes* metabolites revealed a distinct NMR signal with identical chemical shits (1.05 and 2.18 ppm) as propionic acid (a primary *P. acne* metabolite). The 1.05 ppm signal does not overlap with other bacteria metabolites, and its intensity increases linearly with *P. acnes* concentration. Bovine disks injected with *P. acnes* bacteria revealed a very distinct NMR signal at 1.05 ppm, which linearly increased with *P. acnes* concentration.

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Conclusions—The 1.05 ppm NMR signal from propionic acid can be used as a marker of *P. acnes* infection of discs. This signal does not overlap with other disc metabolites and linearly depends on *P. acnes* concentration. Consequently, NMR spectroscopy may provide a non-invasively method to detect disc infection in the clinical setting.

Keywords

P. acnes; biomarker; intervertebral disk; low back pain

Introduction

Vertebral bone marrow lesions known as Modic Changes (MC) strongly associated with Low Back Pain (LBP)[1–7]. According to National Centers for Health Statistics, LBP is the most common disability worldwide, over 26 million US citizens have chronic LBP and up to 43% of these patients (~11 million) have MC[8]. Very often the possible causes of LBP is unknown.

Microbiological studies of excised disc tissues from patients with herniated discs revealed the presence of *Propionibacteria acnes* (*P. acnes)* bacteria in 38–53% of samples[9–11]. While not all studies are in agreement on this controversial topic[12], there is growing amount of evidence that *P. acnes* infection is one of possible cusses of MC1[3]. A positive effect of antibiotic treatment of MC1 patients in a double-blinded clinical trial further supports this theory[13,14]. In addition, newly formed MC1 after a microdiscectomy surgery was associated with the presence of *P. acnes* in disk tissue[11,15]. A recently conducted proteomic analysis of specimens obtained from patients with herniated, degenerated and healthy disks revealed the presence of 56 proteins from *P. acnes*[16]. Importantly, 67 defense-response proteins were found in the tissue of these patients, providing strong evidence of a chronic *P. acnes* infection rather than sample contamination during processing. A direct inoculation of rat and rabbit intervertebral discs with *P. acnes* bacteria leads to the development of MC-like changes and disc degeneration with fibrotic endplate erosion evident by histological examination[17,18].

While *P.acnes* infection is a biologically plausible pathology in LBP patients, antibiotic therapy may not be justified given the detrimental side effects associated with the high doses and long duration shown effective in prior clinical trials. Consequently, there is an urgent need for a reliable and noninvasive method for detection of *P. acnes* in LBP patients and to further investigate the relationship between *P. acnes* and MC.

P. acnes is an anaerobic aerotolerant, Gram-positive, opportunistic pathogen implicated in acne vulgaris, endocarditis, and osteomyelitis (PMID 15634990). *P. acnes* are a part of the normal resident microbiota of the skin, oral cavity and the gastrointestinal and genitourinary tracts[19–21]. These bacteria can also cause prosthetic joint infection[22]. How *P. acnes* seed into the disc and could cause MC1 is unknown, but it was suggested that *P. acnes* enter the bloodstream during innocuous events such as tooth brushing, or through haematogenous spread from a distant septic location[17,23,24]. When disc immunoisolation is violated by disc herniation or endplate damage (both of which are associated with MC1[24]), *P. acnes* may invade the disc as an area with a low oxygen tension and more suitable conditions for a

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proliferation of anaerobic bacteria[11,15]. *P. acnes* utilize anaerobic respiration through succinyl-CoA and produce propionate, a progenitor of propionic acid (PA). PA and inflammatory cytokines, which are produced by disc tissue as a response to *P. acnes* infection, can drain into the bone marrow through endplate defects. This drainage can cause a pro-inflammatory reaction of bone marrow cells and affects bone marrow cell maturation, which was described in MC1[9,10,25].

At present, an infection of the disk with *P. acnes* can only be diagnosed with an anaerobic culture of an excised tissue. A less invasive method of acquiring samples with needle biopsy is unreliable and often produces false negative results. A tool to identify MC patients with infection is critically important for the selection of patients for an antibiotic treatment. It will allow to design definitive studies regarding whether *P. acnes* actually causes infection and eliminate the effect of surgical samples contamination. Furthermore, it would assist with a development of pre-clinical animal models to test the efficacy of antibiotic treatments.

The goal of this study was to demonstrate that *P. acnes* infection of intervertebral discs can be detected noninvasively by NMR spectroscopy. We hypothesized that NMR signal from PA could be used as a biomarker of the infection.

Material and methods

P. acnes isolation

A method for isolating a clinically-relevant strain of *P. acnes* bacteria from a patient was described in our earlier publication[17]. Briefly: an intervertebral disc was surgically removed from a patient with MC1 changes. *P. acnes* was isolated by anaerobic culture of the disc tissue and cryopreserved. Before the injection of an intervertebral disc with *P. acnes* or the extraction of metabolites from *P. acnes*, the bacteria were retrieved from cryostorage and grown under anaerobic conditions in standard tryptic soy broth for 48 hours.

Preparation of bacterial extract for NMR analysis

To identify a biological marker of *P. acnes*, metabolites were extracted from *P. acnes* bacteria. PBS was added to the original *P. acnes* solution (OD600 = 3.0) and different dilutions were prepared: OD600 = 1.50, 0.75, 0.35, 0.175, 0.08 (1/32, 1/16, 1/8, 1/4, 1/2 dilutions, respectively). Metabolites were extracted with methanol/chloroform method[26]. Briefly: the bacterial solutions were suspended in 4 mL of ice-cold methanol in a glass tube. The tube was vortexed for 3–5 min and 4 mL of ice-cold chloroform were added and vortexed again. For the final step, 4 mL of ice-cold water were added and vortexed. The samples were kept at 4° C overnight for the separation of lipids, metabolites and denatured proteins fractions. The liquid metabolite fraction was collected and frozen at −80° C. The frozen samples were lyophilized and dissolved in D2O with known amount of 3-(trimethylsilyl)-[2,2,3,3,-$^{2}H_4$]-1-propionate (TSP). TSP was used as an external concentration reference with known chemical shift (0 ppm).

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Injection of P. acnes into bovine tail disks

Fresh bovine tails were purchased from a local farmer's market. Vertebral discs 2.0–2.5 mm in diameter with surrounding cartilage endplates were excised from the tails. MR imaging and spectroscopy data were first recorded from the uninjected discs tissues. After the control scan, different volumes of the original *P. acnes* solution (OD600 = 3.0) were injected into a middle part of the disc with a 27-gauge insulin syringe. The volumes of the injection varied from 10 to 100 μl. After injection, imaging and spectroscopy data were acquired a second time.

MR imaging and spectroscopy of the disks tissue

Imaging and spectroscopy data of the bovine discs tissue were acquired at preclinical 7T imaging magnet interfaced with Agilent console at the Department of Radiology, UCSF. A 2.5 cm surface transmit/receive RF coil was used for data acquisition. Anatomical images of the specimens were acquired with 3D gradient echo sequence with following parameters: repetition time (TR) = 3 ms, echo time (TR) = 1.5 ms, excitation flip angle = 20°, image resolution 200 μm. 3D-voxel for the spectroscopy data acquisition was positioned in the middle of the disk. The size of the spectroscopy voxel was ~7 × 3 × 10 mm. After the determination of the voxel position, shimming of the B0 magnetic field within the voxel was performed with final line-width of the water signal ~ 30 Hz. Single voxel, Laser pulse sequence with outer water suppression was utilized for the acquisition of the spectroscopy data with TR =2 sec, effective TE = 30 ms and 128 repetitions.

Metabolite extraction from bovine disk tissue

After the acquisition of the spectroscopy data from disc specimens injected with different volumes of *P. acnes*, the tissues were snap-frozen in liquid nitrogen and metabolites were extracted with perchloric acid method[27]. Briefly: frozen tissues were ground at liquid nitrogen temperature in the presence of 6% perchloric acid (3.25 ml per 1 g of tissue). The homogenate was centrifuged at 13,000 rpm for 20 min at 4° C. The supernatant was neutralized to pH 7.0 ± 0.2 with 100 mM KOH. The precipitated salt was removed by centrifugation at 1000 rpm for 5 min and the supernatants were frozen at – 80° C and lyophilized. The metabolic analysis of disks tissue extracts and extracts from different dilutions of *P. acnes* were performed at 500 MHz, high-resolution Bruker NMR spectrometer. Concentrations of PA were calculated based on the integration of NMR signals at 1.05 and 2.2 ppm regions and signal from TSP at 0 ppm. ACD-labs and TopSpin software were utilized for the baseline correction, phasing and the integration.

Results

Detection of propionic acid in bacterial extracts

A final product of the catalytic activity of *P. acnes* bacteria is PA. We hypothesized that this molecule can be used as a marker of *P. acnes* infection. NMR spectra of 13 mM of PA and methanol/chloroform extract of *P. acnes* bacteria (Figure 1A) demonstrate two distinct peaks PA at ~1.05 and ~2.18 ppm (lower spectra). Peaks with identical ppm values were also detected in *P. acnes* extract (Figure 1A; top spectrum). A linear dependence of the 1.05 ppm

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signal intensity on the bacteria concentration was observed (y = x + 0.0103, R^2 = 0.98522) (Figure 1B).

Detection of PA in P. acnes infected disks

In order to demonstrate that *P. acnes* infection can be detected in infected discs, different volumes of the bacteria were injected into bovine disc tissues. NMR spectra of control/uninjected (Figure 2A; bottom) disc, a disc injected with propionic acid (13 mM) (top), and disc injected with *P. acnes* bacteria (middle) show that an additional peak at ~1.05 ppm region is clearly distinct within the spectra from the bovine discs (Figure 2 arrows). A linear dependency of signal intensity at ~1.05 ppm region on the injected volume of *P. acnes* was seen (y = 0.09x + 1.6, R^2 = 0.75121) (Figure 2B).

Quantification of PA concentration in the disk tissue

In order to determine the concentration of PA in the tissues after the administration of *P.acnes* bacteria, we extracted metabolites from the injected discs and quantified PA concentrations with high-resolution NMR spectroscopy. The signal intensity form PA was compared with the reference signal (TSP) and concentrations were determined (Table 1).

Discussion

The goal of this study was to test for the existence of a noninvasive biomarker of *P. acnes* disc infection. We showed that *P. acnes* can be identified by the presence of the 1.05 ppm signal from PA on NMR spectra. Furthermore, we demonstrated the linear dependence of this signal on the concentration of *P. acnes*. The proposed spectroscopic method is a first step for the development of a noninvasive technique to diagnose *P. acnes* infection in patients with LBP.

NMR analysis of P. acnes extracts

PA has two distinct peaks at 1.05 and 2.18 ppm. Peaks with the same chemical shifts were clearly identified on NMR spectrum of metabolites derived from *P. acnes* bacteria. The analysis of NMR data recorded from the different concentrations of *P. acnes* revealed the linear dependence of the 1.05 ppm signal intensity (integral) on the bacterial concentration. We also examined the signal at 2.18 ppm from PA. However, this signal is overlapping with several other metabolites from *P. acnes* and the disc tissue and therefore was more difficult to analyze. We observed a linear dependence of this 2.18 ppm signal on the *P. acnes* concentration but with a different slope (data not shown). Also, 1.05 ppm peak was much easier to identify on the NMR spectra of the infected bovine disc because it does not overlap with other signals from bovine tissues. Therefore, we concentrated our effort on NMR signal at 1.05 ppm region from PA and designated it as a potential biomarker of the *P. acnes* infection.

NMR analysis of bovine disks injected with different concentrations of P. acnes

In order to demonstrate that the NMR signal from PA can be detected in intervertebral discs *in situ*, we performed NMR experiments with isolated bovine discs. Here, the peak at 1.05 ppm region was clearly detected in *P. acnes* and PA injected specimens but not in control/

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uninjected samples. Because the human disc is avascular and its pH may vary with the stage of degeneration, it is important to mention that position of NMR peaks from PA is pH sensitive. The peaks at 1.05 and 2.18 ppm from PA correspond to pH =7.0. A reduction of pH leads to shifting of the signal to lower ppm range. The pH of the injected *P. acnes* solution was approximately 6.0 ± 0.2, therefore, the position of the peak is slightly lower when 1.05 ppm. Yet, the integration of NMR signal from samples injected with the different concentrations of the bacteria revealed the linear dependence of the signal at ~1.05 ppm on the bacterial concentration (Figure 2 B). This was observed despite the fact that extraction of PA from the bovine tissue was a more complicated procedure than metabolites extraction from bacteria solution. The grinding of solid tissue at liquid nitrogen temperature was challenging and produced not very homogeneous powder. We believe this was the main reason for relatively large error bars in the tissue extract experiments. It also can explain slightly different slope and offset of PA NMR signal intensities relative to *P.acnes* concentrations.

Propionic acid concentration measurements in the extract from bovine disks

The concentration of PA produced by *P. acnes* bacteria in MC1 patients is unknown. The lowest concentration of the bacteria tested in this study was 3.0×10^7 CFU/ml. The administration of this amount of *P. acnes* bacteria results in ~83 μmole of PA per gram of the disc tissue. This concentration of PA was readily detectable with our preclinical MRI scanner (Figure 2A, middle spectra). However, the sensitivity of a NMR very much depends on the spectrometer configuration and size of the receive coil. Therefore, we are currently conducting experiments to determine the minimal concentration of PA detectable with available (7T and 3T) clinical NMR scanners. This information will provide a much more realistic estimate of the ability to detect *P. acnes* infection in the clinical setting. However, two facts make us hopeful regarding clinical detection. First, 1.05 ppm signal prom PA was not overlapping with other metabolites from the disc tissue itself. NMR detection of an isolated signal is much easier than the detection of overlapping signals and is limited in theory only by the concentration of the molecule. Second, in our previous studies, we were able to detect signal from lactate at 10–20 μM[27,28]. Even lower metabolite concentrations were detected in projects studying muscle metabolism[29].

The ability to detect *P. acnes* infections with a noninvasive technique will inform the discussion regarding true infection vs. contamination of surgical samples. Furthermore, it will assist with the selection of patients for antibiotic treatment and help with the development of more targeted antibiotic treatments for the patients with *P. acnes* infection. Two clinical trials conducted in which LBP patients were treated with broad-spectrum antibiotics (amoxicillin-clavulanate) yielded favorable results[14,30]. With the rising risk of antibiotic resistance, selective antibiotics instead of broad-spectrum antibiotics should be used whenever possible.

A noninvasive technique would also allow clinical spectroscopy studies on LBP patients without MC and help elucidate the relationship between MC, *P. acnes* infection, and LBP.

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Conclusions

We have shown that *P. acnes* bacteria can be detected within the intervertebral disc using NMR spectroscopy in a pre-clinical model. NMR signal at 1.05 ppm from PA is the most prominent and easy identifiable signal associated with *P. acnes.* The linear dependence of the signal intensity at 1.05 ppm on the concentration of *P. acnes* was detected in experiments with bacteria extracts. Similarly, signal intensity at 1.05 ppm region progresses linearly with the concentration of *P. acnes* bacteria injected into bovine disks. The lowest concentration of *P. acnes* tested in our experiment was 3×10^7 CFU/ml. This concentration of *P. acnes* bacteria produced ~83 μM of PA per gram of tissue, which was sufficient to generate NMR detectable signal in isolated bovine spinal disk.

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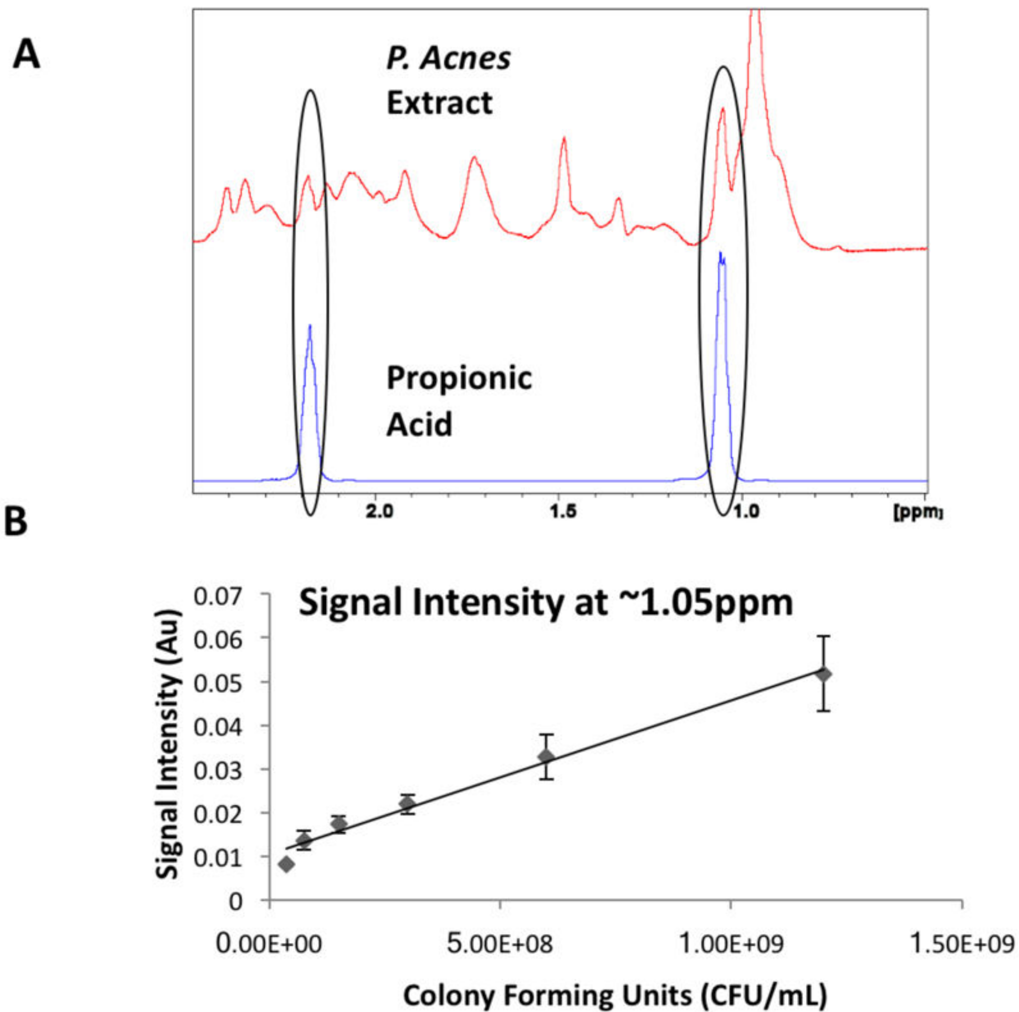

Figure 1.

(A) High-resolution NMR spectra of propionic acid (bottom spectrum) and an extract from *P. acnes* bacteria (top spectrum). Peaks at ~1.05 and 2.18 ppm were detected on both NMR spectra. **(B)** The intensity of the signal at ~ 1.05 ppm as a function of *P. acnes* concentration in bacterial extracts.

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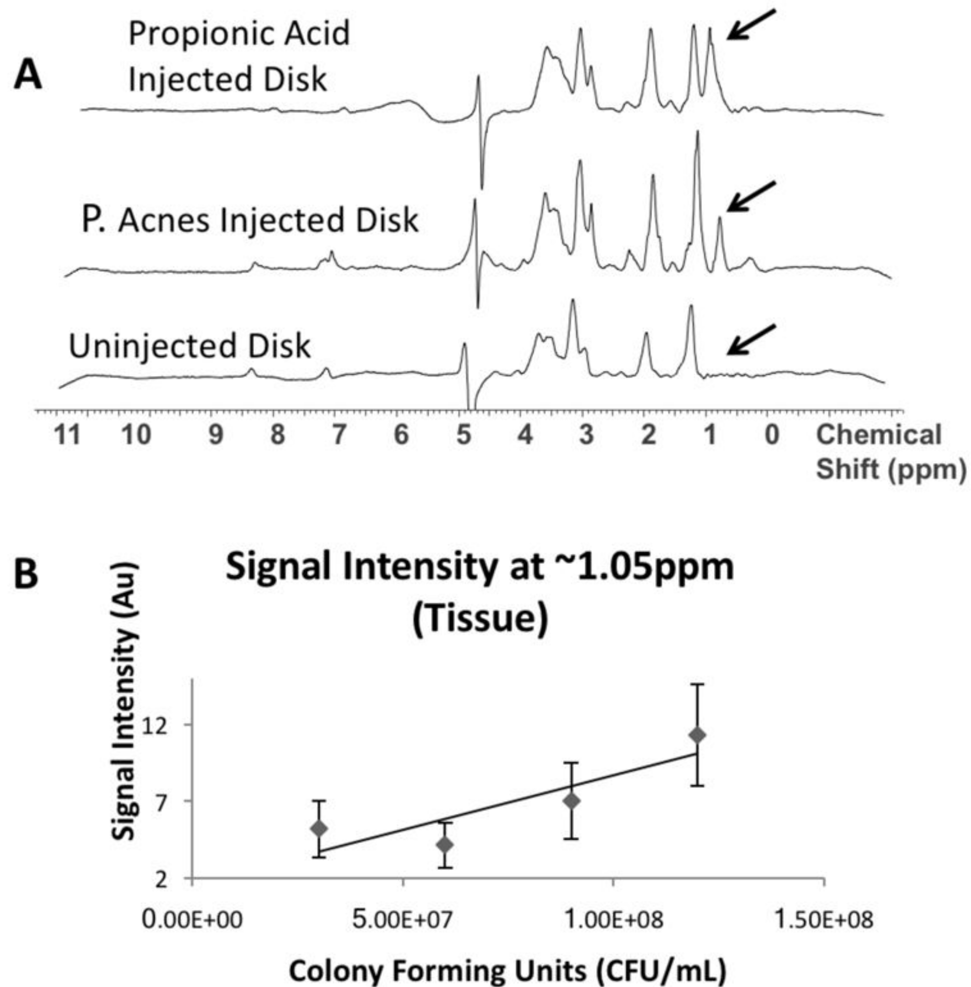

Figure 2.

(A) NMR spectra of an uninjected bovine disk (bottom), disk injected with 25 μl *P. acnes* (middle) and with propionic acid (13mM) (top). **(B)** NMR signal intensity at ~1.05 ppm as a function of *P. acnes* concentration in tissue samples.

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Table 1

Concentration of Propionic acid in a bovine disc tissue extracts after administration of different concentrations of *P.acnes* bacteria.

Colony Forming Units (CFU/mL)	Tissue Extract Concentration (μM/g of Tissue)
3.0×10^{7}	83.04 ± 24.67
6.0×10^{7}	99.45 ±28.71
9.0×10^{7}	130.86 ±47.29
1.2×10^{8}	94.05 ±17.87