Original Article
Polyene Phosphatidylcholine inhibited the inflammatory response in LPS-stimulated macrophages and ameliorated the adjuvant-induced rat arthritis

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Abstract: This study sought to investigate the anti-inflammatory effect of Polyene Phosphatidylcholine (PPC), a clinical drug that is used to treat hepatopathy, on lipopolysaccharide (LPS)-stimulated macrophages and on bovine collagen II-induced arthritis (CIA) rats. In stimulated primary and Raw264.7 macrophages by LPS, PPC significantly down-regulated the relative expression of mRNA such as IL-6, TNF-α, TLR-2, TLR-4, MyD88, and NF-κB while up-regulated IL-10 and TGF-β expression. Moreover, the concentration of IL-6, TNF-α, IL-10, and TGF-β in the cultured supernatants showed the similar tendency with their mRNA alterations. In addition, PPC could significantly inhibit the LPS-induced expression of MyD88 and NF-κB p65 in both mRNA and protein levels. These results suggest that PPC could down-regulate the LPS-stimulated inflammation in macrophages through TLR-2/TLR-4/MyD88/NF-κB pathway in vitro. Furthermore, to explore its effects in vivo, PPC was administrated to CIA rats. In comparison to CIA group, PPC-treated rats showed decreased arthritis score and osteopenia. Besides, PPC exhibited its ability to alleviate the degree of synovial hyperplasia, inflammatory cell infiltration, and destruction of cartilage and bone, thus remarkably improving the condition of CIA rats. In short, this study demonstrated that PPC had the potential to be an anti-inflammatory drug to treat inflammatory disorders such as rheumatoid arthritis.

Keywords: Polyene Phosphatidylcholine, collagen-induced arthritis, macrophages, toll-like receptor

Introduction
Rheumatoid arthritis (RA) is one of the most prevalent autoimmune inflammatory diseases worldwide and is characterized by the infiltration of inflammatory cells and proliferation of fibroblasts in the synovial joints, leading to chronic inflammation and progressive destruction of bone and cartilage [1]. Its pathogenesis is a very complex process involving genetic factors, environmental factors and systemic immune responses [2-4], among which chronic synovial inflammation has been shown to play an essential role in sustainability of the disease [5]. Therefore, down-regulation of synovial inflammation is believed to be an effective way to treat RA.

Synovial tissues contain many types of immunocytes, including synovial fibroblasts (SF), macrophages, dendritic cells, T cells and B cells [6]. The immunocytes augment abundantly during active disease, and their activation has been shown to be the key events that trigger and maintain the inflammatory response [7, 8]. Toll-like receptors (TLRs), as one of the conserved pathogen-associated molecular patterns (PAMPs), play an essential role in this process, revealed by studies in both human and mouse models of RA [9, 10]. Previous studies believed that TLRs are driven by inflammation in response to TLR ligands of microbial origin. However, more recently endogenous TLR ligands have been found in the joints or sera of RA patients and their levels have been correlated with disease activity scores [11, 12]. TLRs therefore have been recognized as key contributors to the pathogenesis of the disease. Hence, there is an increasing interest in targeting TLRs to treat the disease [13]. For example,
Abdollahi-Roodsaz and colleagues had showed that the TLR-4 antagonist had an impressive therapeutic effect in collagen-induced arthritis (CIA) mice [14].

Polyene Phosphatidylcholine (PPC) is extracted from soy and rich in polyunsaturated fatty acid, such as linoleic acid, linolenic acid, and oleic acid. It has been widely used to treat various types of hepatopathy clinically [15], including alcohol-induced hepatic fibrosis [16], hepatocyte steatosis [17], and nonalcholic steatohepatitis (NASH) [18]. Phosphatidylcholine (PC) is the main ingredient of PPC, and is an important component of cytomembrane and organelle membrane. In the past decades, PC had been utilised for protecting health, with its function of nourishing the brain, beautifying the features, reducing weight, and scavenging blood vessels, even viewed as the third nutrient behind protein and vitamine. Interestingly, in recent years, it was reported that several conjugates containing PC could improve the condition of RA [19] and inflammatory bowel disease (IBD) [20], which suggest that PC could act as an anti-inflammatory drug. Thus, we set out to determine the therapeutic effects of PPC on RA. PPC may be easier to practice in clinic, because it has been extensively applied for auxiliary hepatinica treatment for a long time and thus owns a reliable assurance for patients. Nevertheless, it remains obscure whether PPC can treat RA and the possible underlying molecular mechanisms are also unclear.

The present work was to investigate whether PPC could inhibit the inflammatory response in LPS-stimulated macrophages and in CIA rats via TLRs/MyD88/NF-κB pathway. We hope, these results can provide a theoretical basis for the application of PPC in autoimmune and inflammatory diseases including RA.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM, GibCO, USA), fetal bovine serum (FBS, GibCO, USA), streptomycin and penicillin (GibCO, USA), TRizol (Life Technologies, USA), CCK-8 (Dojin-do, Shanghai, China), Annexin V-PE Apoptosis Detection Kit (eBioscience, USA) and mouse ELISA kits (eBioscience, USA), Freund’s complete adjuvant (Sigma, USA), LPS (Sigma, USA), PrimeScript™ RT Master Mix (Takara, Japan), SYBR® Premix Ex Taq™ (Takara, Japan), monoclonal antibodies (MAB) against NF-κB p65 and MyD88 (Abcam, USA), β-actin (Abcam, USA), secondary horseradish peroxidase (HRP)-conjugated antibody (Abcam, USA), Bovine collagen type II (Chondrex, USA), Polyene Phosphatidylcholine (Sanofi Aventis, Spain).

Cell line culture

Mouse macrophage cell line Raw264.7 was obtained from our laboratory and were cultured in DMEM supplemented with 10% FBS, 100 μg/mL streptomycin, 100 U/mL penicillin. Mouse primary peritoneal macrophages were prepared from female C57B/6 mice (6-8 weeks of age) as described [21]. After 2 h, non-adherent cells were removed and the adherent cells were used as peritoneal macrophages. Cell cultures were maintained at 37°C in a humidified atmosphere (5% CO₂).

Detection of cell apoptosis by flow cytometry assay

Raw264.7 cells were treated with indicated concentrations of PPC for 24 h with or without LPS (100 ng/ml), and then harvested and labeled with PE-Annexin V and 7-Amimo-Actinomycin (7-AAD) (Lianke company, China) following the manufacturer's instructions. Stained cells were determined by FACS Canto II flow cytometer (BD, Biosciences, USA) and data were analyzed using FlowJo software.

Cell proliferation assay

A cell counting kit (CCK-8) was used to evaluate cell proliferation according to the manufacturer's instructions. Raw264.7 cells were seeded in 96-well plates at a density of 2×10⁵/well in 100 μl volume. PPC was added in different concentrations (0, 1, 2, 5 and 10 μM). At the point of 20, 44 and 68 h, CCK reagent was added into the medium (10 μl/well). The optical density of each well was determined at 450 nm after 4 h of incubation using a Synergy 2 Microplate Reader (Bio-Tek, USA).

Enzyme-linked immunosorbent assays (ELISA)

The levels of IL-6, TNF-α, TGF-β and IL-10 in cultured cell supernatants were determined using ELISA kits (eBioscience, USA) according to manufacturer instructions.
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Quantitative real-time RT-PCR

RNA was extracted from Raw264.7 cells and peritoneal macrophages using TRIzol reagent, and cDNA was synthesized from the RNA using PrimeScript™ RT Master Mix. Following reverse transcription, cDNA was amplified using SYBR® Premix Ex Taq™ with gene-specific primers. Quantitative PCR analyses were performed in a LightCycler® 480 II detection system (Roche Applied Science, Penzberg, Germany) under the following thermal cycler conditions: one cycle of 5 min denaturation at 95°C, and then 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C for 45 cycles using the primers listed in Table 1. All experiments were performed in triplicate and the Ct values were normalized to endogenous reference (GAPDH). The relative expression of detection indexes of this manuscript was indicated by comparative cycling threshold (Ct) normalized by GAPDH with the 2-ΔΔCt method.

Western blotting analysis

Total protein was extracted from Raw264.7 cells and analyzed with bicinchoninic acid protein concentration assay kit (Beyotime Biotech, Beijing, China). Sample protein was separated by electrophoresis in 10% SDS-PAGE with a Bio-Rad electrophoresis system (Hercules, CA, USA). The primary antibodies (rabbit NF-κB p-65, MyD88 antibody, Abcam, UK; 1:1000 dilutions) were incubated at 4°C 24 h. The secondary anti-bodies (anti-rabbit IgG, 1:2000 dilutions) were incubated for 2 h at room temperature. The membrane containing antibody-protein complexes were visualized with an enhanced chemiluminescence detection system on radiograph film (Bio-rad, Hercules, CA, USA). The bands were scanned and analyzed by the software Quantity ONE (Bio-rad, Hercules, CA, USA). The expression of protein in each sample was normalized by β-actin.

Animals

Female SD rats (125-150 g, 6-8 weeks of age) were purchased from the Animal Center of Xuzhou Medical University (Xuzhou, Jiangsu). All rats were housed in an air-conditioned room at 24°C with a 12 h dark/light cycle and permitted free access to standard laboratory food and water.

CIA model establishment and groups

SD rats were randomly divided into 3 groups with 10 rats in each group: CIA group; PPC treatment group (100 µg/rat) and control group without being modeled. 2.5 ml Bovine collagen type II (5 mg) was dissolved in 2.5 ml Freund’s complete adjuvant, and finally made into an emulsifiable liquid of 1 mg/ml. For arthritis induction, rats were anaesthetized and subcutaneously injected with 100 μl of bovine collagen type II (CII) on day 1 and day 10. Controls received equal volume of Freund’s complete adjuvant in the same time. PPC, at a dose of 100 µg/rat, was administered to CIA rats by tail vein injection on days -1 (the day before the first immunization) and 9.

Arthritis index

The evaluation of arthritis index (AI) mainly involved the hind ankle joints. Four points were selected for each of the hind legs (0 point, no inflammation and joint swelling; 1 point, red spots or mild swelling; 2 points, moderate joint swelling; 3 points, severe joint swelling; 4 points, joint rigidity, deformity or severe dysfunction), and total 8 points for each animal. The degree of joint welling was measured on the basis of the diameter of the right ankle with a vernier caliper.

Table 1. Primer sequences used for quantitative real-time PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>F-CATCTTCTCAATCGAGTGAACA R-TGGGAGTAGAACAGGTACGACCC</td>
</tr>
<tr>
<td>IL-6</td>
<td>F-CACGGCGCTTCTTAC R-AAATGACATCATGTGTG</td>
</tr>
<tr>
<td>TGF-β</td>
<td>F-CTTGTCAGACCTACAGGACT R-TCGTTGTGACGAGCTTCTT</td>
</tr>
<tr>
<td>IL-10</td>
<td>F-GTCAGAGCTCGGGTTTT T-GTTGTGTGACGAGCTTCTT</td>
</tr>
<tr>
<td>TLR-2</td>
<td>F-TGACAGGAAACCTACAGGAGTT R-TTCGATGGAATCGAGTGTG</td>
</tr>
<tr>
<td>TLR-4</td>
<td>F-GAGAAGTGAGTCCTCCCTC R-TCCCATGAAACCTCTAACCAC</td>
</tr>
<tr>
<td>MyD88</td>
<td>F-AAGACAGACAGACACCTCTCTCTC R-AAGAAAGTGAGTCTCCCCTCACGACAGAGTT R-TGGTGTGTGACGAGCTTCTT</td>
</tr>
<tr>
<td>NF-κB</td>
<td>F-GCCGCGGCTTCTTAC R-TGGTGTGTGACGAGCTTCTT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F-GTACATCAGCTGGAAGGGTGAAGG R-ACACAAGGAGGTGAAGGAGGCAGTGTG</td>
</tr>
</tbody>
</table>

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Pathologic evaluation

All animals were sacrificed 42 d after primary immunization. Bilateral hind ankles were fixed with 10% neutral formalin for 48 h, and decalcified with 10% EDTA for 3 weeks. After that, ankles were incised longitudinally, embedded in paraffin. HE staining was used to determine synovial hyperplasia and inflammatory infiltration in the knee joints, and Safranin O-fast green staining was used to evaluate the cartilage degradation while commercial tartrate-resistant acid phosphatase (TRAP) kit was to observe the osteoclastogenesis.

Statistical analysis

All results were presented as mean ± SD. All statistical analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA). Assessments of significant differences comparing multiple groups were performed by one-way or two-way ANOVA. A P-value <0.05 implied significance.

Results

PPC does not induce apoptosis and inhibit growth of Raw264.7 cells

We first tested the apoptotic sensitivity of PPC to Raw264.7 cells in order to determine its cytotoxicity. Figure 1A showed that none of the examined concentrations of PPC (up to 10 μM) induced any apoptosis in LPS-activated Raw264.7 cells at 24 h compared with LPS group (P<0.05). Furthermore, we evaluated whether PPC played an inhibitory role in the growth of Raw264.7 cells using CCK-8 assay. The absorbance values showed that PPC did not reduce the cell viability of Raw264.7 cells within 3 days (Figure 1B, P>0.05). These results suggest that PPC has no cytotoxic effects on Raw264.7 cells.

Figure 1. The effect of PPC on the apoptosis and proliferation of Raw264.7 cells. A: The Raw264.7 cells were exposed to mentioned concentrations of PPC in the absence or presence of LPS (100 ng/ml) for 24 h, and then the apoptosis profiles were determined by FACS. B: The Raw264.7 cells were exposed to mentioned concentration of PPC for 24, 48, 72 h, and then their proliferation were monitored by CCK-8. Data are shown as mean ± SD of three independent experiments. Comparisons among multiple groups were done using two-way ANOVA. *, P<0.05, **, P<0.01.
PPC inhibits the release of pro-inflammatory cytokines induced by LPS in macrophages

Macrophages are well known to play a crucial role in the regulation of inflammatory response. Therefore, this study used Raw264.7 cells and primary macrophages as a model to investigate the anti-inflammatory function of PPC. The effects of PPC on the expression of cytokines, no matter at the level of mRNA or protein, both in LPS-stimulated Raw264.7 cells and primary macrophages were evaluated. As shown in Figure 2A, the mRNA expression of TNF-α and IL-6 in Raw264.7 cells were significantly decreased in LPS plus PPC group compared with LPS stimulated group (P<0.05). These results show that PPC could potently inhibit LPS-induced production of pro-inflammatory cytokines IL-6 and TNF-α. With regards to anti-inflammatory factors, however, the mRNA expression of TGF-β and IL-10 were significantly increased (P<0.05). Moreover, the expression of these cytokines at protein level shared the similar tendency with their mRNA alternations (Figure 2C). In addition, PPC also inhibited IL-6 and TNF-α production while concurrently promoting IL-10 production in primary macrophages (Figure 2B). Overall, PPC can down-regulate the inflammatory response to LPS.

PPC inhibits the activation of TLR-2/TLR-4/MyD88/NF-κB pathway stimulated by LPS in the macrophages

TLR-4/TLR-2 ligation leads to recruitment of its downstream signaling pathways including the adapter protein MyD88 and transcription factor NF-κB, which ultimately result in the production of pro-inflammatory cytokines. Hence, this study examined the expression of TLRs/MyD88/NF-κB both at mRNA and protein levels. As shown in Figure 3A, the mRNA expres-
sion of TLR-2, TLR-4, MyD88 and NF-κB in LPS stimulated Raw264.7 cells were significantly reduced by PPC (P<0.05). Moreover, a similar regulatory role of PPC on these genes in mouse primary macrophages was also observed (Figure 3B, P<0.05). In addition, western blot analysis (Figure 3C) also showed that PPC could reduce LPS-induced activation of MyD88 and NF-κB p65. Therefore, these results suggest that PPC can decrease LPS-induced activation of MyD88 and NF-κB signaling pathway triggered by TLR-2/TLR-4 in macrophages.

**PPC effectively ameliorates the condition of rat CIA**

Significant swellings of CIA groups had been mimicked in the right and left knee joints (Figure 4A). Both arthritis score and the thickness of paws and ankles in PPC treatment group were significantly lower compared with those of CIA group (Figure 4A, P<0.05). The erythema and swelling in the toes and ankles of rats were significantly lessened (Figure 4B) after PPC treatment. Moreover, the loss of sclerotin, destruction of structural integrity and narrowing of the joint space were also improved after PPC treatment (data not shown).

HE staining showed that articular surface of rats in the control group were smooth, and there weren’t any infiltration inflammatory cells in the articular cavity (Figure 5A). However, the abnormal hyperplasia was identified, accompanying with a large number of inflammatory cells infiltration, in the synovial tissues of rats from the CIA group (Figure 5B), while both of which were attenuated when injecting PPC (Figure 5C). Safranin O-fast green is able to put color on the articular cartilage. Compared with the control group (Figure 5D), the articular cartilage of CIA rat became less and thin, and the
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Figure 4. The therapeutic effect of PPC on rat CIA. Bovine CII was subcutaneously injected to induce the arthritis model on day 1 and day 10. Controls received only Freund’s complete adjuvant in the same time. 100 µg PPC was intravenously administered to each CIA rat on day 1 and day 9. The arthritis scores, the thicknesses of rat paw and ankle thickness (A) were evaluated from day 0 to day 27 (n=10 for each group). An example of improved condition of CIA rat joints was presented in (B). Comparisons among multiple groups were done using two way ANOVA. Control group vs CIA group, *, P<0.05, **, P<0.01. PPC treatment group vs CIA group, *, P<0.05, **, P<0.01.

Discussion

The immunocytes infiltrating in the RA joint produce a large number of cytokines, such as TNF, IL-1 and IL-6, thus creating the inflammatory environment [22, 23]. Biological therapies such as the utilization of TNF antibody and IL-6 receptor antibody to treat RA are being recommended in recent years [24, 25]. Although there are improvements in many aspects by the use of these methods, some shortcomings indeed exist. For instance, many patients, rather than achieving an expected curative effect adequately after anti-TNF treatment, may only become unresponsive to this treatment over time. Moreover, the fancy price of biological therapies remains a factor that severely restricts their use. Therefore, current studies should still insist on developing novel therapeutic approaches to RA treatment. This study mainly evaluated the anti-inflammatory effects of PPC on LPS-stimulated macrophages and on CIA rats to explore its potential to be a drug candidate against RA.

PPC did not obviously affect the growth and apoptosis of Raw264.7 cells, suggesting a weak cytotoxicity to normal cells. Actually, the safety is obvious, because the drug has been widely applied to treat liver diseases for many years. Also, PC is the fundamental substance of cell and is often recommended as health remedy. Thus, PPC was proposed to have considerable exploration potential to be an ideal anti-inflammatory drug.

PPC could inhibit the release of pro-inflammatory cytokines (TNF-α, IL-6) stimulated by LPS and boost the production of anti-inflammatory cytokines (IL-10, TGF-β). Previous studies had illustrated that joint damage of RA patients was mainly caused by the infiltration of mononuclear cell, proliferation of synovial fibroblasts, and the accumulation of matrix metalloproteinases.
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and proinflammatory cytokines [26, 27]. On one hand, TNF-α and IL-6 could promote progressive destruction of cartilage and bone, regulate synovial hyperplasia, and mediate bone resorption via enhancing the synthesis of rheumatoid factor [28-30]. On the other, the anti-inflammatory cytokines IL-10 and TGF-β limit the inflammatory response by inhibiting pro-inflammatory cytokines, suppressing the differentiation of Th1 cells and inducing regulatory T cells, however, the levels of these anti-inflammatory cytokines were declined in the active stage of RA [31-34]. Therefore, it is important to control the disease progression by regulating the production of the cytokines. In brief, the present study showed the regulatory function of PPC on Raw264.7 cells and primary macrophages, which seemed to be one reason for ameliorating the severity of CIA rats.

Moreover, PPC was also found to inhibit LPS-induced activation of TLR-2 and TLR-4/MyD88/NF-κB pathway which participate in the whole process of RA. Macrophages from patients in the active stage of RA could express higher levels of TLR-2 and secrete more inflammatory cytokines than those from inactive state [35, 36], which result in exacerbation of arthritis.

Figure 5. The pathological manifestations of CIA rats after PPC treatment. All animals were sacrificed at day 42 after primary immunization. Ankle joints were harvested for evaluating the pathological changes. A-C: Showed the representative results of HE staining. A: The articular surface was smooth without any infiltration of inflammatory cells in the articular cavity of control group. B: The infiltration of inflammatory cells and the unlimited proliferation of SF cells in the articular cavity of CIA rats. C: The articular surface was smooth without invading SF cells after PPC treatment. D-F: Showed the results of Safranin O-fast green staining. D: The bone structure and cartilage of control group were intact in articular cavity. E: The articular cartilage encountered serious destruction in CIA rats. F: The condition of destruction was ameliorated by PPC treatment. G-I: Showed the results of TRAP staining. G: Only a few osteoclasts were found in control joints. H: The numbers of osteoclasts were significantly increased in the joints of CIA rats. I: The numbers of osteoclasts were obviously decreased after PPC treatment.
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Similarly, TLR-4 could accelerate joint inflammation and bone erosion [37]. Notably, increasing studies demonstrated that many endogenous TLR ligands appeared at different stages of RA [38], which revealed the key contributions of TLRs to the inflammation [39]. In fact, targeting TLR-2 and TLR-4 have showed an attractive therapeutic effect on CIA mouse models and RA patients [40]. A previous study had reported that PC exclusively interacted with TLR-2 and TLR-4 [41]. Our study found that PPC effectively downregulated the expression of TLR-2 and TLR-4 in LPS stimulated macrophages.

Another pro-inflammation mechanism involved in RA is the activation of MyD88 and NF-κB, which strongly increases the expression of pro-inflammatory cytokines and chemokines [42]. MyD88, the central adaptor molecule interacting with all TLRs except TLR3, is involved in the spontaneous production of cytokines in RA [43]. Moreover, factor(s) released from synovial membrane cultured from RA tissues could stimulate macrophages in a MyD88-dependent way [44]. This study found that PPC significantly inhibited the expression of MyD88. Furthermore, PPC was also demonstrated to reduce the expression of NF-κB, which was also observed in the liver tissue of NASH rats [17]. Consequently, the inhibitory effect of PPC on the LPS-induced inflammatory cytokines might pass through down-regulating the TLR2/TLR4/MyD88/NF-κB pathway.

RA patients present some features including persistent infiltration of leukocytes, synovial membrane hyperplasia and destruction of bone and cartilage [45, 46], which are all important factors of RA pathological and clinical manifestations. Aggressive infiltration of leukocytes in rheumatoid synovial tissue produces large amounts of cytokines and chemokines, and even causes synoviocyte hyperplasia, invades cartilage, and finally lead to the destruction of bone and cartilage. Synoviocyte hyperplasia results in pannus formation and damage of the joints [47] while osteoclast activation mediates abundant bone resorption and destroys bone and cartilage, ultimately resulting in malformation and even dysfunction of joints [46]. Pathological observation of our study revealed that PPC could potently alleviate the condition of CIA rats by decreasing inflammatory cell infiltrate, ameliorating synovial hyperplasia and progressive destruction of articular cartilage.

In summary, this study showed that PPC, the classic hepatina in clinic, inhibited LPS-induced inflammation in macrophages via down-regulating TLR-2/TLR-4/MyD88/NF-κB pathway in vitro, and thus improving the condition of CIA rats. These results indicate that PPC could act as an anti-inflammatory drug to treat inflammatory diseases, including RA.

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Disclosure of conflict of interest

None.

Authors’ contribution

Conceived and designed the experiments: WP, KYZ, RXT. Performed the experiments: WP, WTH, HWX, XYL, XML, FFS, HL. Analyzed the data: WP, WTH, XYL, FFS. Contributed reagents/materials/analysis tools: SPQ, RXT. Wrote the manuscript: WP, WTH.

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