Initial Responses of Articular Tissues in a Murine High-Fat Diet-Induced Osteoarthritis Model: Pivotal Role of the IPFP as a Cytokine Fountain

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Abstract

Obesity and high body mass index are associated with a higher incidence of osteoarthritis (OA). The aim of this study is to investigate the involvement of the infrapatellar fat pad (IPFP) in the sub-acute effect of a high fat diet (HFD) on the development of knee-OA. C57BL/6J male mice were fed either a HFD or a normal diet beginning at seven weeks of age. Tissue sections were evaluated with immunohistological analysis. The IPFP was excised, and mRNA expression profiles were compared using real-time RT-PCR analysis. Osteoarthritic changes were initiated in the HFD group after eight weeks of the HFD. Increased synovial cell number and angiogenesis at the anterior edge of the tibial plateau were exhibited prior to osteophyte formation. Quantitative histological analysis indicated that osteophyte volume was significantly increased in the HFD group after eight weeks, along with an increase in the IPFP volume, the size of individual adipocytes and the number of vessels in the IPFP. Histomorphometrical analysis revealed osteophyte area was significantly associated with IPFP area, individual adipocyte area and vascular area. Real-time RT-PCR analysis demonstrated elevated mRNA expression of inflammatory cytokines, growth factor, and adipokines in the IPFP after eight weeks of the HFD. These findings are in parallel with increased expression of the CD68 macrophage marker after eight weeks of the HFD. Expression levels of the adipokines were significantly correlated with expression of TNF-α, VEGF and TGF-β. Immunohistological analysis revealed that the Nampt protein was highly expressed in the IPFP especially around the site of osteophyte formation. Apoptosis and proliferation of chondrocytes were both enhanced at the site of osteophyte formation, indicating higher cell turnover at this region. These observations suggest the IPFP plays a pivotal role in the formation of osteophytes and functions as a secretory organ in response to a HFD.

Introduction

Osteoarthritis (OA) is a chronic degenerative joint disorder characterized by articular cartilage destruction and osteophyte formation, and is prevalent in society as a major cause of disability. OA risk factors identified by previous epidemiologic studies are limited to age, trauma history, occupation, gender and obesity [1]. Obesity and high body mass index are associated with a higher incidence of OA [2–5].

Excess weight caused by obesity introduces increased weight bearing on the knee joints, implicating the influence of mechanical factors in the development of OA especially in major joints of the lower extremity [6]. Trauma, joint instability, and developmental dysplasias are also recognized as predisposing factors in animal models of OA [1]. A number of cohort studies have demonstrated that obesity is an independent risk factor for hand OA [7,8]; however, mechanical stress cannot explain such a correlation. Therefore, it has been hypothesized that one or more systemic factors are responsible for the correlation between obesity and OA.

Obesity is also associated with an increased amount of adipose tissue, which is recognized as having potent endocrine activity and can give rise to inflammation. Adipose tissue expresses and secretes a variety of bioactive peptides, known as adipokines, which act at both the local (autocrine/paracrine) and systemic (endocrine) level [9].

Activation of adipose tissue macrophages within fat depots is also accompanied with the development of an obesity-induced proinflammatory state [10,11]. Chronic inflammation triggered by obesity is associated with several diseases such as type 2 diabetes, defective immunity, hypertension, atherosclerosis and several cancers [12]. These studies suggest that inflammatory molecules secreted from adipose tissue may provide a critical connection between obesity and OA.

The infrapatellar fat pad (IPFP) is located in the knee underneath the patella, between the patellar tendon, femoral condyle and tibial plateau, and is positioned closely to the synovial layers and cartilage surfaces of the knee joint. The IPFP contains...
adipocytes and has an increased number of immune cells such as lymphocytes, monocytes and granulocytes that have migrated from the blood circulation [13]. IPFPs from OA patients contain inflammatory cytokines, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF) alpha, and interleukin (IL) 6 [14]. Thus, the IPFP could play an important role in the initiation and progression of knee-OA. However, the precise roles of the IPFP at the initiation of OA, for instance, or whether a HFD initiates the IPFP to produce more inflammatory mediators, has not been elucidated.

Activation of the synovial layer (synovitis) is seen in many osteoarthritic joints and formation of osteophytes at the junction of the periosteum and synovium is a common feature [2] [3]. This process is initiated with the formation of chondrocytes, followed by chondrocyte hypertrophy. Finally, chondrocytes develop into osteophytes as a result of ossification. However, it is unknown what initiates these processes.

Various laboratories have established in vivo OA models in order to study the mechanisms of OA development. [1,15–18] [19–21] Providing a HFD has been shown to increase the incidence of OA in male mice of C57Bl6 strain [19,20]. In order to investigate the mechanisms of OA initiation, the initial reaction of the knee joints in response to a HFD was evaluated. A detailed histological investigation has been employed to permit rapid evaluation of the murine knee joints as a consequence of a HFD. Histological grading analyses for assessment of OA were utilized rather than quantitative analyses, as there is a lack of measurable markers for OA.

Taken together, we hypothesized inflammatory responses would occur in the IPFP in advance of the initiation of OA. To clarify the role of the IPFP, we induced OA with a HFD and investigated the initial responses of the knee articular cartilage and the IPFP by detailed histological analysis and real-time RT-PCR analysis.
Materials and Methods

Male C57BL/6j mice were purchased from Sankyo Labo (Tokyo, Japan). Ethical approval was obtained from the institutional review board of Tokyo Medical and Dental University. C57BL6j mice were fed a diet containing 32% fat for the HFD group or 4.8% fat for the control group (HFD32 and CE-2; CLEA Japan, Inc. Tokyo, Japan) [22] from the age of seven weeks. All of the animals were allowed unrestricted activity and were provided food and water ad libitum. None of the mice died during the experimental period.

Assessment of OA Severity

Mice were sacrificed at four, eight, and twelve weeks after initiating the diet (n = 10 at each time point). Whole knee joints were removed by dissection, fixed in 4% paraformaldehyde, and decalcified in EDTA. After dehydration and paraffin embedding, serial 5-μm sagittal sections were made from the whole medial compartment of the joint. Three sections (from lanes 1–3, Figure 1a, b) were obtained at 100-μm intervals from the weight-bearing region of each knee joint. Lane1 was defined as the section in which the central region of the medial meniscus was continuous (Figure 1b, arrow). Lane2 and Lane3 were 100 μm and 200 μm lateral to Lane1 respectively. The sections were stained with Safranin O–fast green or HE. OA severity in the tibial plateau was evaluated according to a cartilage destruction score (1). Quantitative osteophyte determination was made in the sections from lane 1 (Figure 1a, b) using Image-Pro Plus 4.1 software (Media Cybernetics, Carlsbad, CA). The protruded region, which stained green by Safranin-O staining (Figure 2g–i), was defined as bony osteophyte and quantified.

Histomorphometry Analysis

Histomorphometric measurements were performed using image analysis software (Image Pro Plus 4.1, Media Cybernetics, Carlsbad, CA, USA). Based on immunostaining for anti-CD31 antibody, immunopositive cells were defined as endothelial cells and the vascular areas were quantified. For quantification of adipocytes, over 30 adipocytes in IPFP were selected and the area of each cell was quantified and averaged.

RNA Extraction and Real-time RT-PCR

The IPFP tissue was excised using a surgical microscope and microsurgical technique at the previously indicated periods. Total RNA was extracted from the IPFP using TRIzol according to the manufacturer’s directions (Invitrogen). Real-time RT-PCR was performed using the SuperScript III Platinum Two-Step qRT-PCR kit with SYBR Green on the Mx3000P QPCR System. Briefly, 0.5 μg total RNA was incubated with 10 μl 2× RT reaction mix and 2 μl RT, and then incubated for 50 min at 42°C. The reaction was terminated by incubating for 5 min at 85°C. The cDNA mixture was then incubated for 30 min at 55°C in the presence of RNase H. The PCR reaction was carried out using a mixture of Platinum SYBR Green qRT-PCR Super-Mix UDG, the template cDNA, 10 mM of the primer mix, and DNase-free H2O with a total volume of 20 μl per well. The cycling conditions were performed as indicated in the Invitrogen SuperScript™ III Platinum two-step qRT-PCR kit with SYBR Green. Gene expression was normalized to the endogenous control GAPDH, and fold changes in the genes of interest were determined using the comparative threshold cycle (Ct) method.

Immunohistochemistry

The protein expression of CD31, Nampt, PCNA or TGF-β1 was examined by immunohistochemistry with anti-mouse CD31 antibody, anti-mouse Nampt antibody, anti-mouse PCNA antibody (Abcam Biochemicals, Cambridge, UK), or anti-mouse TGF-β1 antibody (Santa Cruz Biotechnology, INC.) used according to the manufacturer’s instructions. Briefly, tissue sections were incubated overnight at 4°C with primary antibodies, followed by a 30-min incubation at room temperature with appropriate secondary antibodies. Next, the signal was visualized using peroxidase-conjugated avidin and diaminobenzidine from a Vectastain kit, according to the manufacturer’s instructions (Vector Laboratories, Burlingame, CA).

TUNEL Assay

The TUNEL assay was performed using a TUNEL detection kit according to the manufacturer’s instructions (Takara Shuzo, Kyoto, Japan). A section was procured from lane 2 (Figure 1a, c) of each specimen and incubated with 15 μg/ml of proteinase K for 15 min at room temperature, then washed with Phosphate Buffered Saline (PBS). Endogenous peroxidase was inactivated with 3% H2O2 for 5 min at room temperature. After washing with PBS, sections were immersed in buffer containing deoxynucleotidyl transferase and biotinylated dUTP and incubated for 90 min at 37°C in a humid atmosphere. After washing in PBS, signals were examined by fluorescence microscopy and the number of TUNEL-positive cells in the articular cartilage above the tidemark was determined.

Statistical Analysis

Data are expressed as the mean ± 1 SD. Statistical analysis was performed with the Mann-Whitney U test. P values less than 0.05 were considered significant. Pearson linear regression was used to determine the degree of association between mRNA expression of adipokine or inflammatory cytokine and histological values. The linear regression coefficient R2 were reported. Values of P<0.05 were accepted as significant.

Results

Impact of HFD on IPFP Histology Over Time

Mice fed the HFD weighed 10% more than normal diet mice by four weeks, 20% more by eight weeks and 50% more by twelve weeks (Figure 2c, p<0.05). Histological examinations of the slides from Lane 3 were made to estimate the effect of the HFD on IPFP histology (Figure 2a, b). The total volume of the IPFP was increased in the HFD group (Figure 2b, d) when compared to the normal diet group (Figure 2a). Average individual adipocytes found in the IPFP were also significantly increased from week eight of the HFD diet when compared to the control group mice (Figure 2e). Concurrently, active angiogenesis was observed in the IPFP of the HFD group (Figure 2f).

Figure 3 depicts features of osteophyte formation at high magnification. The IPFP volume was slightly increased in the HFD group by week four (Figure 3d). Inflammatory features, including enhanced angiogenesis (arrows) and infiltration of macrophage-like round synovial cells (asterisk) were observed at the anterior edge of the tibial cartilage by week four in the HFD group (Figure 3d, e). Cartilaginous osteophytes gradually developed in the same region (Figure 3e, h, i, arrowhead), and the features of synovitis continued by twelve weeks (Figure 3f). Ossification of cartilaginous osteophytes was apparent, with size and maturity increasing from eight weeks to twelve weeks of the HFD (Figure 3f, i, arrowhead, Figure 4a). Osteophytes formed...
predominantly at the antero-medial region of the tibial plateau, so that the size of the osteophyte was larger in Lane 1 compared to Lane 2 or 3 (data not shown). Histological evaluation indicated the cartilage destruction score of the HFD group was significantly increased after 8 weeks of the HFD (Figure 4b, \( P<0.05 \)).

To elucidate the implication of adipogenesis and angiogenesis in osteophyte formation, the correlations between histological values and osteophyte volume were evaluated. As shown in Figure 4c, osteophyte area was significantly associated with IPFP area, individual adipocyte area and vascular area. Correlation of these values (\( R^2 = 0.5923 \) for osteophyte area and IPFP area, \( R^2 = 0.6358 \) for osteophyte area and adipocyte area, \( R^2 = 0.4376 \) for osteophyte area and vascular area) were strong.

mRNA Profiles in the IPFP

We evaluated the expression levels of inflammatory cytokines in the IPFP for the HFD and control groups, since the IPFP has recently been implicated in the pathology of osteoarthritis [13,23]. The IPFP was excised using a surgical microscope and microsurgical technique at eight and twelve weeks after initiation of the diet. Real-time RT-PCR analysis revealed the expression levels of adipokines (Leptin, Nampt, Chemerin and Lipocalin2), inflammatory cytokines (VEGF and TNF-\( \alpha \)), and growth factor (TGF-\( \beta \)) were significantly elevated in the IPFP from the eighth week of the HFD. Simultaneously, the CD68 macrophage marker was increased in the IPFP from the eighth week of the HFD (Figure 4d). The expression of adiponectin, which is reported to play a protective role in OA [24], was not affected by HFD both in IPFP and serum (Figure 4d).

To elucidate the association between adipokines and inflammatory cytokines, the correlation coefficient among their expression levels were calculated. As shown in Figure 4a, adipokine expression and TNF-\( \alpha \), VEGF, and TGF-\( \beta \) were significantly correlated. In addition, CD68 expression positively associated with Nampt expression, indicating the influence of macrophages on Nampt expression.

The expression of NAMPT is increased in the plasma and synovial fluid of patients with OA [25]. Immunohistological analysis for Nampt was performed to verify the spatial and temporal expression pattern of Nampt. Immunohistological examination revealed that Nampt protein was highly expressed in the IPFP at the twelfth week of the HFD (Figure 5a, b). Of note, Nampt expression was condensed in the vicinity of osteophyte formation (Figure 5a, b).

**Figure 2.** Histological analysis of the IPFP of mice fed a HFD and a normal diet. Sections of articular cartilage from control (a) and HFD (b) mice stained with HE. Note advanced angiogenesis (arrows) and hypertrophic adipocytes in (b). (c); HFD mice weighed 10% more than normal diet mice by four weeks, 20% more by eight weeks and 50% more by twelve weeks. (d–f); Histological quantification of the IPFP area (d), average individual adipocyte area (e) and total vascular area (f) in IPFP. \( * = P<0.05 \) using the Mann-Whitney U test. doi:10.1371/journal.pone.0060706.g002
Immunostaining for PCNA, a marker for proliferation, was conducted to estimate cell turnover activity at the site of osteophyte formation. PCNA positive cells were abundantly observed at the site of osteophyte formation in the HFD group (Figure 5d). These observations indicated an enhanced state of cell turnover at this region.

Chondrocyte Apoptosis in HFD Models

Apoptotic cells exist abundantly at the chondro-osteophyte, which is observed at the peripheral area of osteoarthritic joints, even in the early stage of the disease [26]. Chondrocyte apoptosis is increased in OA cartilage and is anatomically linked to proteoglycan depletion [27]. These observations prompted us to investigate the effect of the HFD on chondrocyte apoptosis. TUNEL staining was performed for the lane 2 section in the HFD mice and controls at week eight of the diet. TUNEL-positive cells were abundantly observed in the superficial layer of the articular cartilage (Figure 5c) and at the site of osteophyte formation in the HFD group (Figure 5d). These observations indicated an enhanced state of cell turnover at this region.

Combined with enhanced proliferation of chondrocytes in this region, the knee joint in the HFD group is in the course of osteoarthritic change, including osteophyte formation.

Discussion

Our findings revealed that a HFD induces hypertrophy of the IPFP in association with inflammation in less than three months time. In order to objectively quantify the severity of OA development in the HFD model, we evaluated serial sections for histological findings. We quantified the severity of OA development using osteophyte volume and the number of TUNEL-positive cells rather than scoring of the visual findings. Several laboratories have succeeded in developing murine knee OA with more than a three-month exposure to a HFD. OA susceptibility for HFD mice depends on mice strain and gender [28]. For example, mice of strain Dba respond less readily to the HFD than mice of strain C57 black [28]. Furthermore, female mice are less susceptible to the change in dietary regimen than males. Male mice of strain C57 black fed a diet containing 29% fat from the age of six or twelve months to the end of their lives...
showed an accelerated onset and an increased incidence of OA as compared with control mice fed a stock diet containing 5% fat [19]. Our study reveals the initiation of OA change occurs at an earlier period than previously reported.

We demonstrated enhanced angiogenesis, infiltration of macrophage-like cells and increased synovium at the anterior edge of the tibial cartilage in advance of and in combination with osteophyte formation by week eight of the HFD. We also found enhanced adipokine secretion with IPFP hypertrophy, followed by aggregation of synovial cells. The IPFP has recently been implicated as an additional joint tissue involved in the development and progression of knee-OA [13,23]. The human osteoarthritic knee IPFP was found to contain significantly elevated protein levels of inflammatory cytokines and adipokines [14]. These inflammatory mediators have been found in synovial fluid and have been suggested to influence cartilage and synovial metabolism [29]. Our study demonstrated that the mRNA expression levels of inflammatory cytokines, such as VEGF and TNF-α, and adipokines, such as Leptin and Nampt, and growth factors, such as TGF-β, were enhanced at week eight of the HFD which is consistent with previously reported data [14,25,29,30]. We clearly exhibited adipocyte hypertrophy and increased angiogenesis were strongly correlated with osteophyte volume (Figure 3c). Furthermore, the expression of adipokines (Nampt and Leptin) and adipocyte hypertrophy markers (Lipocalin2 and Chemerin) was correlated with expression of TGF-β and inflammatory cytokines in the IPFP (Figure 4a). These results indicate adipocyte hypertrophy closely links osteophyte formation through secretion of inflammatory cytokines.

Leptin has been detected in synovial fluid (SF) obtained from patients with OA [30]. Leptin expression is also enhanced in both osteophyte and cartilage tissue obtained from patients with OA. Leptin is reported to act as a pro-inflammatory adipokine with a catabolic role in cartilage metabolism via the up regulation of proteolytic enzymes [31]. However, Leptin but not Adiponectin promoted the expression of cartilage-specific markers through mitogen-activated protein kinase, Janus kinase and phosphatidylinositol-3 kinase signaling pathways [32]. The metabolic function
of Leptin may play a pivotal role in osteophyte formation considering the enhanced expression level of leptin in the IPFP.

The expression of NAMPT is increased in the plasma and synovial fluid of patients with OA [25]. Although NAMPT has been reported to be produced by chondrocytes from OA patients, our study has demonstrated the highly enhanced expression of Nampt in the IPFP in response to a HFD and in controls. Nampt protein expression was increased at the IPFP in the HFD. PCNA positive cells were abundantly observed at the peripheral region of osteophyte formation in the HFD group. (c, d); Analysis of apoptosis in TUNEL-stained knee joint sections from HFD and control mice. The number of TUNEL-positive cells was increased in knee joint cartilage from mice in the HFD group especially at the areas with osteophytes (triangle) and the superficial layer of articular cartilage (arrows). (e, f); The number of TUNEL-positive cells per section in the superficial layer of the articular cartilage was determined under fluorescence microscopy. Reported values are the mean ± 1 SD of five mice per group. * = P < 0.05 by Mann-Whitney U test.

doi:10.1371/journal.pone.0060706.g005

Figure 5. Correlation between adipokines and inflammatory cytokines or CD68 and immunohistological evaluation of knee joints.
(a); Correlation between mRNA expression of adipokines, such as Leptin and mRNA expression of Nampt, and CD68 or inflammatory cytokines. The expression of Leptin and Nampt were positively correlated with TNF-α, VEGF and TGF-β. Nampt expression was strongly correlated with the macrophage marker CD68. (b); Immunostaining for Nampt and PCNA in the IPFP from week twelve of the HFD and in controls. Nampt protein expression was increased at the IPFP in the HFD. PCNA positive cells were abundantly observed at the peripheral region of osteophyte formation in the HFD group. (c, d); Analysis of apoptosis in TUNEL-stained knee joint sections from HFD and control mice. The number of TUNEL-positive cells was increased in knee joint cartilage from mice in the HFD group especially at the areas with osteophytes (triangle) and the superficial layer of articular cartilage (arrows). (e, f); The number of TUNEL-positive cells per section in the superficial layer of the articular cartilage was determined under fluorescence microscopy. Reported values are the mean ± 1 SD of five mice per group. * = P < 0.05 by Mann-Whitney U test.

doi:10.1371/journal.pone.0060706.g005

Adipose tissue macrophage infiltration during obesity. We observed that the CD68 macrophage marker was increased in the IPFP at week eight of the HFD and that this occurred simultaneously with the enhancement of Nampt and TNF-α expression. Nampt mRNA level is strongly correlated with the CD68 macrophage-specific marker and TNF-α mRNA levels in adipose tissues [34]. In this study, we report a strong correlation between mRNA expression of Nampt and CD68 in the IPFP (Figure 4a). TNF-α is a pro-inflammatory cytokine produced mainly by macrophages and lymphocytes. It is also produced by adipose tissue although the expression level is low in humans [35]. Large-scale studies of gene expression using micro-array approaches have revealed that variations in gene expression in white adipose tissues (WAT) are essentially related to a macrophage infiltration in WAT of obese mice [10]. Thus, locally present CD68-positive macrophages may play important roles in the augmentation of these cytokines. Also, studies have shown an increased inflammatory response associated with the presence of hyperleptinemia without obesity [36,37], and that leptin is able to
control TNF-α production and activation by macrophages [36]. We have shown TNF-α expression to be significantly associated with Namp and Leptin expression (Figure 4a). These observations suggest Leptin may also regulate TNF-α expression in the IPFP.

The expression of Chemerin and Lipocalin2 was enhanced in the IPFP by HFD (Figure 4c). Chemerin is predominantly expressed in adipocytes and promotes calcium mobilization and chemotaxis of immature dendritic cells and macrophages [38] In 3T3-L1 adipocytes, Chemerin expression is enhanced during differentiation [39]. Human plasma levels of Chemerin were significantly associated with body mass index, circulating triglycerides, and blood pressure [39], indicating a potent function of Chemerin in immune and metabolic homeostasis. Lipocalin2 is a 25 kDa glycoprotein expressed in neutrophil granules, adipocytes and chondrocytes [40–42]. Lipocalin2 belongs to the large family of Lipocalins which have high affinity for small hydrophobic ligands such as steroids and LPS [42]. Lipocalin2 expression was dramatically enhanced in adipocytes by IL-1β treatment [43]. In the analysis of cartilage degradation and protein release using proteomics, Lipocalin2 is identified as a biomarker of cartilage degradation [44]. Overexpression of Lipocalin2 in chondrocytes caused reduction in proliferation and promotion of hypertrophy [41]. These observations suggest potent roles of Chemerin and Lipocalin2 in the inflammatory responses of the IPFP and enhanced chondrocyte apoptosis in the HFD group.

On the other hand, Adiponectin levels in plasma and the IPFP were not affected by HFD in our study. Adiponectin has been shown to be implicated in the pathogenesis of osteoarthritis [42]. Plasma Adiponectin levels were reported to be significantly higher in patients with OA than in healthy controls [45]. Conversely, Adiponectin concentrations in plasma and synovial fluid show significant inverse correlation with disease severity, suggesting a possible protective role of Adiponectin in OA [24]. Perhaps an extended time course of the HFD may alter Adiponectin levels in the IPFP.

We showed that TGF-β mRNA expression was gradually enhanced by a HFD. The ability of TGF-β to induce osteophyte formation was previously demonstrated [46,47]. There is significant overlap in the location of TGF-β-induced and experimental OA-induced osteophyte formation [46]. These observations confirm that TGF-β plays a role in osteophyte development during experimental OA [48]. Leptin, which is upregulated in parallel with TGF-β in this study, stimulates chondrocyte synthesis of TGF-β in animal experiments [30]. Thus, Leptin may act as a trigger to stimulate TGF-β expression from the IPFP. Consistently, we observed TGF-β mRNA expression was positively correlated with Leptin expression in the IPFP (Figure 4a).

It is still unclear whether the events observed in the IPFP are directly induced by HFD or are an indirect response to the destruction of articular cartilage. For instance, gain of weight in HFD mice may result in an increase in mechanical load and trigger the wear of cartilage, followed by local inflammation. To elucidate this problem, further experiments will be required.

In conclusion, we have shown that articular cartilage degradation and osteophyte formation can be triggered from as early as eight weeks of a HFD with detailed evaluation methods. Our observations suggest pivotal roles for the IPFP in the development of osteophyte formation and cartilage degradation. Furthermore, our methods gave the ability to adjust and reproduce the episodes of applied mechanical loading and metabolic alteration. This provided an opportunity to investigate articular cartilage responses to metabolic stress and the mechanisms involved in the progression of OA.

Author Contributions
Conceived and designed the experiments: YA. Performed the experiments: MI. Analyzed the data: MI. Contributed reagents/materials/analysis tools: YH MT AO. Wrote the paper: YA MI.

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