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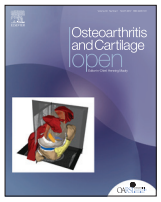

Intra-articular injection of inorganic pyrophosphate improves IL-1β-induced cartilage damage in rat model of knee osteoarthritis in vivo

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ABSTRACT

Objective: Osteoarthritis (OA) is the most common form of chronic joint disease, affecting mainly the elderly population. This disorder is caused by cartilage degeneration with complex changes in the chondrocyte phenotype. Inorganic pyrophosphate (PPi) was shown to counteract the detrimental effect of interleukin (IL)-1β challenging in an in vitro OA model based on rat articular chondrocytes. It also maintained the differentiated articular phenotype, mostly by down regulating wingless-related integration site (Wnt)-5a secretion. These observations suggest a PPi protective role for chondrocyte in vitro.

Methods: To address this hypothesis in vivo, we investigated the impact on knee joint of three intra-articular injection (IAI) of PPi in a rat model of cartilage damage induced by IAI of IL-1β, where cartilage degradation and synovial inflammation are similar to that observed in OA. Cartilage and synovial membrane were collected after 7 days of challenge by IL-1β.

Results: PPi was able to reduce the deleterious effect of IL-1β. This effect was observable on the expression of cartilage extracellular matrix metabolism markers and confirmed by histology with safranin O and hematoxylin-eosin-saffron (HES) staining. Inorganic pyrophosphate also repressed the Wnt5a expression induced by IL-1β. No effect was observed on the inflammatory response of the synovial membrane.

Conclusion: These results demonstrate that PPi improves IL-1β-induced cartilage damage in rat but not the associated inflammation of synovial membrane. Thus, PPi could become a molecule of interest to restrict the progression of this disorder.

1. Introduction

Osteoarthritis (OA) is the most common form of joint disease, which leads to severe disability and loss of quality of life. It involves not only hyaline articular cartilage but the entire joint including the nearby muscles, the underlying bone, the ligaments, the synovial membrane, and the capsule [1]. The world health organization scientific group on rheumatic diseases estimated that 10 % of the population, with an age over 60 years old, has significant clinical problems which can be attributed to OA [2,3]. This joint disorder is histologically characterized by cartilage degeneration and loss, and episodes of synovial inflammation [4–7].

Hyaline articular cartilage consists of differentiated cells called chondrocytes which maintain the homeostasis of their own surrounding extracellular matrix (ECM). ECM is mainly composed of collagens, such as type II collagen (Col2), and proteoglycans (PGs), such as large chondroitin sulfate PGs like aggrecan and versican. When mature and functional, healthy chondrocytes express highly Col2 and aggrecan genes, while Col1 and versican are faintly expressed [8–11].

The cartilage degeneration caused by OA results, among others, from a loss of chondrocyte phenotype towards hypertrophy with a recapitulation of endochondral ossification [12,13]. Insulin-like growth factor binding protein (IGFBP)-3 is an OA marker which binds IGF-1, a major growth factor taking part in the maintenance of differentiated chondrocyte phenotype with collagen and aggrecan synthesis, ECM metabolism, and cartilage integrity. OA causes an augmentation of IGFBP-3 which triggers chondrocyte hypo responsiveness to IGF-1 and extinguish its actions [14,15]. Chondrocyte dedifferentiation is observed with an increase of Col1 and versican expression in parallel with a decrease of

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Col2 and aggrecan [8,16,17]. Although OA mechanisms are not fully understood, a joint low-grade inflammation is involved in establishing this disease. The inherent production of pro-inflammatory cytokines, such as interleukin (IL)-1β, promotes joint tissue inflammation, e.g., synovial inflammation with swelling and immune cell infiltration [18,19]. Cyclooxygenase (COX)-2, microsomal prostaglandin E_2 synthase (mPGES)-1, and tumor necrosis factor (TNF)-α belong to the mediators of inflammation induced by IL-1 β and when released in joint space, contribute to cartilage degradation [19–22].

IL-1β participates in OA progression by influencing chondrocytes and inducing their dedifferentiation [23,24]. This pro-inflammatory cytokine promotes hyaline ECM catabolism by stimulating the secretion of matrix degrading enzymes, such as matrix metalloproteinase (MMP)-1 and MMP-3 [8,25]. In addition, IL-1β prevents ECM production by inhibiting the synthesis of its components, including Col2 or aggrecan [8,18]. This cytokine also increases the expression of wingless-related integration site (Wnt)5a, which is strongly involved in rabbit cartilage degradation in vitro, while is genetic paralog Wnt5b remains undetected [26,27]. However, Wnt5a and Wnt5b are elevated in OA patients, which suggests their possible influence in OA progression even if they seem to have different mechanisms of action. Indeed, Wnt5a is able to exacerbate joint degeneration by inducing inflammatory cytokines, whereas Wnt5b is not [27].

It was previously demonstrated in a rat in vitro OA model with chondrocytes exposed to IL-1β that the loss of differentiated phenotype could be induced by decreasing the transport of inorganic pyrophosphate (PPi). This effect is subject to the activation of the canonical Wnt pathway in a Wnt5a-dependent manner. However, it could be partially reversed by increasing extracellular PPi (ePPi). These observations are in favor of an ePPi protective role for chondrocyte to counterbalance the deleterious effect of Wnt5a. This protective outcome also concerns the proteins involved in ePPi production, such as inorganic pyrophosphate transport regulator (Ank) and ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1) [28,29].

Intra-articular injection (IAI) as a means for local delivery of drugs or active molecules to the joint cavity is a method to reach hyaline articular cartilage. IAI of IL-1β is a suitable in vivo rodent model to cause an inflammation responsible for cartilage damages similar to that found in OA [30–34], which can be scored for cartilage degeneration according to the Osteoarthritis Research Society International (OARSI) recommendations [35–37].

The aim of our work was to investigate if PPi could counteract the in vivo adverse effect of IL-1β leading to cartilage damage with features similar to OA in the same way as in vitro. In this study and for the first time, the impact of an IAI of PPi on knee joint was evaluated in a rat model of cartilage damage induced by IAI of IL-1β. To evaluate the potential PPi effect, knee hyaline cartilage and synovial membrane were analyzed. A single IAI of PPi was shown to improve cartilage damage in rat knee joint by maintaining hyaline ECM and differentiated chondrocyte phenotype, but had no influence on synovial inflammation. Overall, these results demonstrated that PPi could become a molecule of interest to be formulated for IAI route as a treatment to preserve cartilage and counteract OA progression.

2. Method

Reagents were obtained from Merck (Saint-Quentin-Fallavier, France), and media components were obtained from Lonza (Colmar, France), unless specified otherwise.

2.1. Animals

Male Wistar rats (150–175 g), obtained from Charles River (L'arbresle, France) were acclimated for 1 week in the laboratory before experimentation. Animals were housed in groups of 3 or 4 in solid-bottomed plastic cages, with access to tap water and standard pelleted rodent chow (Scientific Animal Food and Engineering) ad libitum. Room temperature was set at 23 °C (± 1 °C), and animals were subjected to a 12-h cycle of light/dark. All experiments were performed in accordance with national animal care guidelines and were preapproved by the local ethics committee (CELMEA of Université de Lorraine, protocol code: APAFIS#6624-2016042215241254 and date of approval: October 10, 2016) who approved the experimental protocols, and guidelines for laboratory procedures were followed at all times. Animal's injections and necropsy were performed while the animals were under general anaesthesia with the volatile anaesthetic isoflurane (AErrane; Baxter, Guyancourt, France).

2.2. Experimental design

Rats were injected in both knee joints. IAI of IL-1β (Tebu-bio, Le Perray-en-Yvelines, France) was used to trigger knee OA. Rat joints with "IL-1β" condition developed OA features by injecting 1 μg of IL-1β (1 μg/knee) in 50 μL of saline per knee (20 ng/μL) at day 0, day 2, and day 5 as described by van Beuningen et al. [38]. For the "Control" condition, the three injections (50 μL/knee) were made of saline alone. The PPi effect was evaluated with a single dose on healthy and OA knee rats with the "PPi" and "IL-1β + PPi" conditions respectively. The "PPi" condition meaning a single dose of 0.1 mM of PPi (freshly prepared as a mixture of $Na_2H_2P_2O_7$ and $Na_4P_2O_7$; pH 7.4) in saline (50 μL/knee) at day 0, followed by two saline injections at day 2 and day 5. The "IL-1β + PPi" condition meaning a single dose of 0.01 mM of PPi associated with 1 μg of IL-1β in saline (50 μL/knee) at day 0, followed by two IL-1β injections (1 μg in 50 μL of saline) at day 2 and day 5. The necessary sampling for the four investigated conditions ("Control", "IL-1β", "PPi", and "IL-1β + PPi") was made at the end of the experimental procedure (day 7).

After three injections at 0, 2 and 5 days, total knee joints, knee synovial membranes, and knee hyaline cartilage (patellae, femoral condyles, and tibial plateaus) were collected at day 7. Synovial membranes, femoral condyles, and tibial plateaus were immediately frozen at −80 °C for RNA extraction then PCR analysis. Patellae were processed for ex vivo incorporation of $Na^{35}SO_4$ into cartilage and 1,9-dimethylmethylene blue (DMMB) assay. Total knee joints and synovial membranes were also conserved for histological investigation.

2.3. Histology and OARSI scoring

Knee joints and synovial membranes from injected rats were fixed for 24 h in 4 % paraformaldehyde immediately after sacrifice. Knee joints were decalcified in EDTA for 1 month, then further fixed in 4 % paraformaldehyde. The samples were embedded in paraffin. Paraffin sections (5 μm) were deparaffinized in Tissue Clear (Bayer Diagnostics, New York, United States) and rehydrated in a graded series of ethanol. For histological examination, sagittal knee sections were stained for PGs with Safranin O/fast green and synovial membranes with hematoxylin-eosin-saffron (HES) for fibrous and cellularity aspects. Cartilage damage was evaluated by the OARSI 2010 scoring method for both tibias and femurs. All slides were scored in double blind by two observers to mitigate technician variability. For each knee, 10 sections were exploited. 10 knees were collected per time point and per group (4 groups).

2.4. Proteoglycan synthesis ex vivo

Cartilage explants (patellae) were placed for 3 h at 37 °C in a humidified 5 % CO_2 atmosphere with 0.6 μCi/mL $Na_2^{35}SO_4$ (PerkinElmer, Villebon sur Yvette, France) in RPMI HEPES 1640 medium supplemented with 2 mM L-glutamine, 100 μg/mL streptomycin, and 100 IU/mL penicillin. After 5 washings in saline, explants were fixed overnight at room temperature in 0.5 % cetylpyridinium chloride dissolved in 10 % formalin buffer. Explants were then decalcified for 6 h in 5 % formic acid and dissolved in 0.5 mL Soluene (Packard, Rungis, France) overnight at 42 °C. The radioactivity reflecting the ^{35}S content of each individual explant was measured in disintegrations per minute by liquid scintillation counting (Packard, Rungis, France).

2.5. Glycosaminoglycan determination

The DMMB dye assay was used to assess sulfated GAG production at the 7-day endpoint with modifications of the previously described method [39]. The DMMB (Aldrich, Milwaukee, WI) solution was prepared and stored at room temperature in an amber bottle. Patellae were digested with 60 μg of Papain for 2 h at 60 °c. Samples were assayed in duplicate by mixing 0.1 mL of sample with 1.25 mL DMMB solution. For standards, 25 μL of 0–100 μg/mL chondroitin sulfate were added to 250 μL DMMB solution. All tubes were incubated at room temperature in the dark for 30 min followed by absorbance read at 525 nm. Sample concentrations were determined from the standard curve and results expressed as μg of sulfated GAGs/mL.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Femoral condyles and tibial plateaus were harvested at day 7, then pooled for RNA extraction. Explants were decalcified for 18–24 h with EDTA 165 mM pH = 7.4 in RNA later (Ambion, Huntingdon, U.K.) before separation of the cartilage layer from the underlying bone. Total RNAs from synovium and explants were isolated using a commercially available phenol-chloroform solution (Trizol). Integrity of RNA pool was verified by electrophoresis. One hundred ng of total RNAs were reverse transcribed for 90 min at 37 °C in a 20 μl reaction mixture containing 10 mM dNTP, 5 μM random hexamer primers, 1.5 mM $MgCl_2$, and 200 U Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, France). cDNAs production was performed in a Mastercycler gradient thermocycler (Eppendorf, France). Next, real time PCR was performed by the Step One Plus (Applied Biosystems, France) technology using specific primers (Table 1) and iTAQ SYBRgreen master mix system (Biorad, France). All reagents used for Reverse transcription-polymerase chain reaction were added at the concentrations recommended by the manufacturer. Melting curve was performed to determine the melting temperature of the specific PCR products and, after amplification the product size was checked on a 1 % agarose gel stained with Gel Red (Biotium, Interchim, France) as quality control. Each run included positive and negative reaction controls. The mRNA levels of the gene of interest and of the *ribosomal protein 29* (*RP29*), chosen as housekeeping gene, were determined in parallel for each sample. Quantification was determined using the ΔΔCt method and the results were expressed as fold change over the appropriate control.

2.7. Statistical analysis

Results are expressed as the mean ± standard error of the mean. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software). The Shapiro-Wilk test was used to determine the normality of our data. Statistical analyses were done using either one-way ANOVA followed by Bonferroni's posttest or unpaired *t*-test, with Welsh' correction when variances were significantly different. The p values less than 0.05 were considered significant.

3. Results

The increase of PPi in an in vitro OA model based on rat chondrocytes exposed to IL-1β participates in maintaining cell phenotype, indicating a protective role for PPi against dedifferentiation [28,29]. The in vivo impact of PPi on cartilage damage induced by IAI of IL-1β in rat knee joint was investigated in the present study.

The influence of PPi was evaluated by examining its potential effect on cartilage ECM, chondrocyte phenotype, OA marker and Wnt pathway, and synovial inflammation.

3.1. Inorganic pyrophosphate protects the cartilage of rat knee joint from IL-1β-induced proteoglycan loss

After experimental procedure, total rat knee joints were collected for histology analysis. The obtained knee tissue sections were stained with Safranin O/fast green staining to evaluate the PG content in articular hyaline cartilage. Safranin O staining intensity of histological sections (red staining) is associated with PG content, whereas fast green is used to counterstain the tissue (green staining) (Fig. 1A, left). The "Control" condition, showed a healthy articular hyaline cartilage with homogeneous thickness and high intensity staining. In the "PPi" condition, cartilage was similar to the "Control" condition, suggesting that PPi is a harmless molecule in healthy rat joint at this concentration. On the contrary, the "IL-1β" condition displayed typical OA features with cartilage cracks, a decreased cartilage thickness revealing an extensive loss of chondrocytes and ECM, and a lack of Safranin O staining indicating PG depletion. The "IL-1β + PPi" condition demonstrated that PPi counteracts the progress of cartilage damage by slowing the propagation of cracks and by rescuing ECM loss, particularly PGs (Fig. 1A, left).

The stained tissue sections were also scored according to OARSI recommendations [35] to quantify the histological observations (Fig. 1A, right). No significant difference was found for the "Control" and "PPi" conditions with a score between 0 and 1, meaning no cartilage degeneration to a minimal degeneration (5-10 % of the total projected cartilage area affected by chondrocyte or ECM loss). The score for the "IL-1β" condition was significantly higher compared to the "Control" condition and was greater than 3, meaning a moderate cartilage degeneration (26-50 % affected) for the in vivo rat model used after 7 days of knee cartilage challenged by IL-1β. A significant PPi effect on the "IL-1β + PPi" condition was noticed compared to "IL-1β" used alone with a score decrease closed to 2 (mild degeneration, 11-25 % affected). This confirmed PPi protectiveness to lessen IL-1β deleterious impact on PG loss (Fig. 1A, right).

Table 1
Sequences of specific primers for RT-PCR analyses. ACAN—aggrecan; Ank—inorganic pyrophosphate transport regulator; Col1—type I collagen; Col2—type II collagen; Cox-2—cyclo-oxygenase type 2; IGFBP3—insuline like growth factor binding protein 3; Enpp1—ectonucleotide pyrophosphatase/phosphodiesterase 1; MMP-1—matrix metalloproteinase type 1; MMP-3—matrix metalloproteinase type 3; mPGES-1— microsomal prostaglandin E_2 synthase-1; TNF-tumor necrosis factor; Wnt5a—wingless-related integration site a; Wnt5b—wingless-related integration site 5b; Fwd—forward primer; RT-PCR—real-time polymerase chain reaction; Rev—reverse primer; RP—ribosomal protein.

Genes	Sequences 5′–3′
ACAN	Fwd: CAA-CCT-CCT-GGG-TGT-AAG-GA
	Rev: TGT-AGC-AGA-TGG-CGT-CGT-AG
Ank	Fwd: CAA-GAG-AGA-CAG-GGC-CAA-AG
	Rev: AAG-GCA-GCG-AGA-TAC-AGG-AA
Col1	Fwd: TTG-ACC-CTA-ACC-AAG-GAT-GC
	Rev: CAC–CCC–TTC-TGC-GTT-GTA-TT
Col2	Fwd: TCC-CTC-TGG-TTC-TGA-TGG-TC
	Rev: CTC-TGT-CTC-CAG-ATG-CAC-CA
Cox-2	Fwd: TAC-AAG-CAG-TGG-CAA-AGG-CC
	Rev: CAG-TAT-TGA-GGA-GAA-CAG-ATG-GG
IGFBP3	Fwd: GTT-CCA–CCC–CCT-CCA-TTC-AA
	Rev: TTT-GTA-GCG-CTG-GCT-GTC-TT
Enpp1	Fwd: TAT-GCC-CAA-GAA-AGG-AAT-GG
	Rev: GCA-GCT-GGT-AAG-CAC-AAT-GA
MMP-1	Fwd: CAC-CAA-TCA-GTT-CAA-CGC-AGA
	Rev: ACC-AGG-TAC-TAG-AAT-CAC-GGG
MMP-3	Fwd: TCT-GGG-CTA-TCC-GAG-GTC-AT
	Rev: TGC-ATC-GAT-CTT-CTG-GAC-GG
mPGES-1	Fwd: ACC-CTC-TCA-TCG-CCT-GGA-TA
	Rev: ATG-CGT-GGG-TTC-ATT-TTG-CC
RP29	Fwd: CTC-TAA-CCG-CCA-CGG-TCT-GA
	Rev: ACT-AGC-ATG-ATT-GGT-ATC-AC
TNF-α	Fwd: AGC-CCT-GGT-ATG-AGC-CCA-TGT-A
	Rev: CCG-GAC-TCC-GTG-ATG-TCT-AAG-T
Versican	Fwd: CAC-CAC-CTC-TGA-GTG-GGT-TT
	Rev: TGT-GTG-GGC-TGC-ATT-TGT-AT
Wnt5a	Fwd: GGC-TCA-TGG-CAT-TTA-CCA-CT
	Rev: AAA-TAG-GCA-GCC-GAG-AGA-CA
Wnt5b	Fwd: AAC-GTG-GAG-TAT-GGC-TAC-CG
	Rev: TGA-TCC-CTT-GGC-AAA-GTT-CT

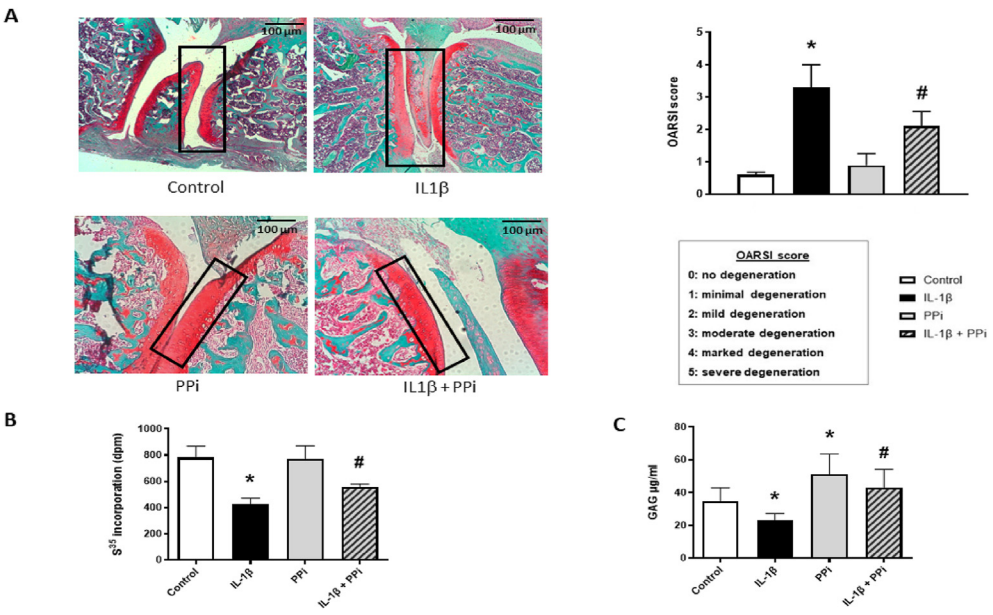

Fig. 1. Evaluation of the inorganic pyrophosphate effect on the proteoglycan content and the degeneration of articular cartilage in a rat model of knee cartilage damage induced by IL-1β. (**A**) Evaluation of cartilage PG content and degeneration in knee joint tissue sections. Left pictures: representative images of cartilage PG content in knee joint tissue sections highlighted by Safranin O/fast green staining (black frame). The black scale bar corresponds to 100 µm. Right panel: cartilage degeneration due to IL-1β-induced damage was estimated in rat knee joint tissue sections according to OARSI score. Among PGs, sulfated GAGs produced by chondrocytes were measured in patellae (**B**) by ex vivo incorporation of ^{35}S-sulfate and (**C**) by DMMB dye assay. The reported data are represented as mean ± SEM (n = 12 rats). Significance is indicated as * when compared to Control and # when compared to IL-1β ($p < 0.05$). DMMB—1,9-dimethylmethylene blue; GAG—glycosaminoglycan; IL—interleukin; OARSI— osteoarthritis research society international; PG—proteoglycan; PPi—inorganic pyrophosphate; SEM— standard error of the mean.

Among PGs, sulfated glycosaminoglycans (GAGs) produced by chondrocytes were estimated in patellae by ex vivo incorporation of ^{35}S-sulfate and by DMMB dye assay (Fig. 1B and C respectively). The histological results found in Fig. 1A were corroborated by these two methods.

Patellar GAG anabolism was measured for 3 h (Fig. 1B). No difference for neosynthesized sulfated GAGs was observed between the "Control" condition and the "PPi" condition. Anabolism was reduced by 47 % in the "IL-1β" condition compared to the "Control" condition. However, the negative effect of IL-1β was counteracted by adding PPi with only a 26 % decrease of anabolism for the "IL-1β + PPi" condition (Fig. 1B). DMMB dye assay was used to measure the concentration of the total sulfated GAGs (Fig. 1C), PPi used alone showed a positive effect with a 52 % increase in GAG synthesis compared to the "Control" condition, while the "IL-1β" condition still had a negative impact with a 31 % decrease. The PPi addition in the "IL-1β + PPi" condition restored GAG synthesis at a concentration similar to the "Control" condition (Fig. 1C). Both methods showed a PG depletion with "IL-1β" that was hampered in the "IL-1β + PPi" condition thanks to the PPi action which seems to participate in PG maintenance.

3.2. *Inorganic pyrophosphate partially protects the differentiated phenotype of rat articular chondrocyte in vivo*

The phenotype of chondrocytes isolated from femoral condyles and tibial plateaus was assessed by measuring gene expression. The cartilage of "Control" rats was composed of healthy chondrocytes with an articular hyaline phenotype. The selected genes were coding for ECM components (Col1, Col2, aggrecan, and versican) and ECM degrading enzymes (MMP-1 and MMP-3) (Fig. 2).

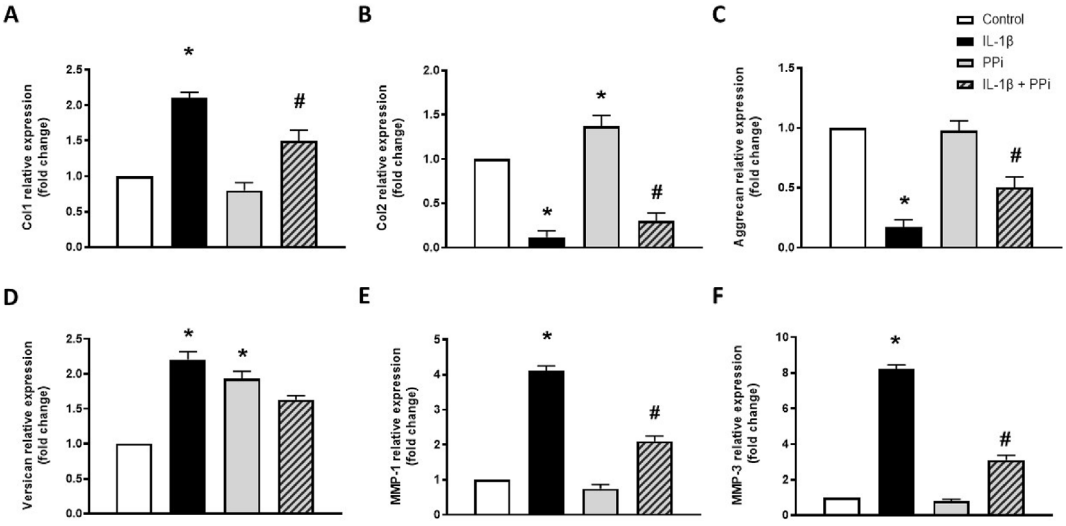

Fig. 2. Evaluation of the inorganic pyrophosphate effect on the chondrocyte phenotype in a rat model of knee cartilage damage induced by IL-1β. The relative abundance of transcript for (**A**) type 1 collagen (Col1), (**B**) Col2, (**C**) aggrecan, (**D**) versican, (**E**) MMP-1, and (**F**) MMP-3 was normalized to RP29. The reported data are represented as mean ± SEM (n = 12 rats). Significance is indicated as * when compared to Control and # when compared to IL-1β ($p < 0.05$). Col1—type I collagen; Col2—type II collagen; IL—interleukin; MMP-1—matrix metalloproteinase type 1; MMP-3—matrix metalloproteinase type 3; PPi—inorganic pyrophosphate; RP—ribosomal protein; SEM— standard error of the mean.

PPi used alone had no effect on the selected genes except for Col2 and versican with a 1.5- and 2-fold overexpression respectively compared to the "Control" condition (Fig. 2B).

"IL-1β" used alone showed chondrocytes with a disturbed phenotype compared to the "Control" condition by substantially repressing the hyaline markers Col2 and aggrecan with a 82 % and 71 % decrease respectively (Fig. 3B and C), while the dedifferentiation markers Col1 and versican were increased by 52 % (Fig. 2A and D). In addition, IL-1β enhanced the ECM catabolic response by a 4- and 8.2-fold increase of MMP-1 and MMP-3 respectively (Fig. 3E and F).

The data obtained with the co-injection of PPi with IL-1 β confirmed the results of the previous sections. PPi improved chondrocyte phenotype compared to the "IL-1β" condition by decreasing Col1, versican, MMP-1, and MMP-3 (29 %, 27.5 %, 53 % and 62 % respectively, Fig. 2A, D, 2E, and 2F) and by increasing Col2 and aggrecan (3- and 3.25-fold respectively, Fig. 2B and C). This positive effect was particularly strong to reduce catabolic markers and helped to partially restore the expression of hyaline markers.

3.3. Inorganic pyrophosphate hinders IL-1β induced OA marker and Wnt pathway in rat articular chondrocyte in vivo

To continue the study of the PPi impact on the chondrocytes in a rat model of knee cartilage damage induced by IAI of IL-1β, the expression of other genes was monitored for the four experimental conditions: the OA marker IGFBP-3 as well as the Wnt pathway (Wnt5a and Wnt5b) and proteins involved in its control (Ank and Enpp1) (Fig. 3).

IL-1β used alone induced a 4.85-fold augmentation of IGFBP-3 expression compared to the "Control" condition, while PPi alone caused a repression of 54.2 %. This confirms that IL-1β participates actively in OA establishment. On the contrary, PPi co-injected with IL-1β triggered a 58.3 % decreased expression (Fig. 3A). Wnt5a expression exhibited the same profile as IGFBP-3 with a 2.53-fold overexpression with "IL-1β" alone and a 43.1 % reduction in the "IL-1β + PPi" condition (Fig. 3B). The results obtained for IGFBP-3 and Wnt5a support the PPi positive effect on chondrocytes by alleviating cartilage damage and inhibiting Wnt5a pathway.

Wnt5b expression remained unaffected, whatever the experimental condition, which suggests its lack of implication in the studied in vivo model (Fig. 3C).

Ank and Enpp1 are involved in the PPi machinery. Their expressions displayed almost the same profile with no difference in the "PPi" condition and a strong decrease (53 %) in the "IL-1β" condition compared to the "Control" condition (Fig. 3D and E). However, Ank expression remained unaffected by the PPi addition in the "IL-1β + PPi" condition (Fig. 3D), contrary to the one of Enpp1, which was restored (Fig. 3E). These results suggest a major contribution of Enpp1 compared to Ank in the PPi positive effect on articular cartilage.

3.4. Inorganic pyrophosphate does not affect the IL-1β-induced inflammation of rat synovial membrane

After evaluation of the PPi effect on the IL-1β-induced cartilage damage in rat knee joint, another articular tissue was examined to determine if PPi can influence other parts of the joint. Consequently, rat synovial membranes were also tested to study the PPi effect on inflammation with HES staining of tissue sections to highlight immune cell infiltrations and with gene expression of specific markers (COX-2, mPGES-1, and TNF-α) (Fig. 4).

HES staining showed that compared to the "Control" condition, PPi alone had no effect on the morphology of synovial membrane, whereas the immune response due to IL-1β alone caused a noticeable fibrosis and cellular invasion associated with a pannus. Synovial membrane had the same aspect as the one in the "IL-1β" condition when PPi was co-injected with IL-1β, indicating that PPi had no influence on synovial inflammation (Fig. 4A).

These results were corroborated by gene expression analysis. The inflammation markers COX-2, mPGES-1, and TNF-α were overexpressed (4.2, 5.3, 2.1-fold respectively; Fig. 4B, C, and 4D) by IL-1β induction when the pro-inflammatory cytokine was used alone compared to the "Control" condition. No significant difference was detected between the "Control" and the "PPi" conditions, and between the "IL-1β" and the "IL-1β + PPi" conditions (Fig. 4B, C, and 4D). The latest observations confirmed the previous histological examination, demonstrating that PPi showed no effect to counteract the synovial inflammation induced by IL-1β.

Thus, the capacity of PPi to protect cartilage from IL-1β-induced degeneration is not transferable to hinder IL-1β induced synovial membrane inflammation.

4. Discussion

It was estimated that OA is the most common musculoskeletal disease which affects more than 300 million patients worldwide. It leads to cartilage degeneration in parallel with the establishment of a joint low-grade inflammation. The mechanisms causing this joint affliction are still under study since they remain not fully understood and to preserve the patient's quality of life, the current therapeutic strategy focuses on treating the symptoms that are characterized by pain and impairment of

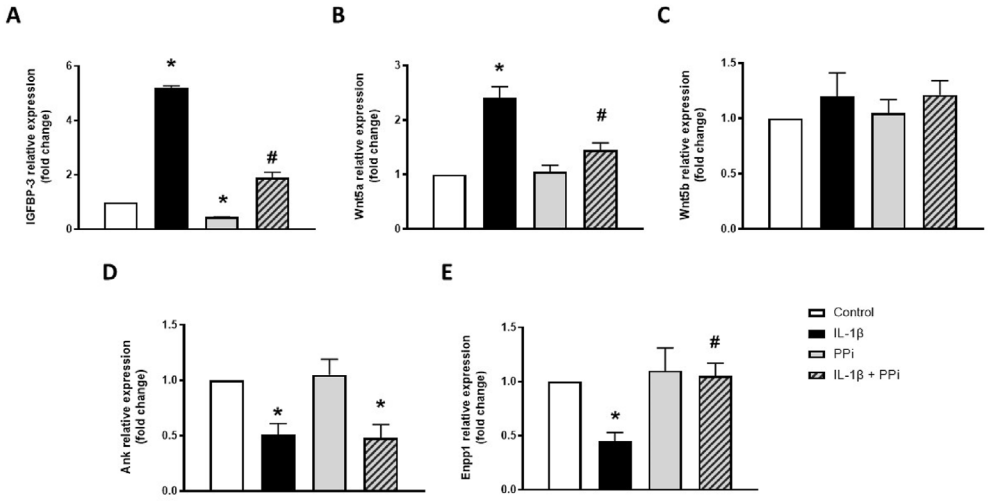

Fig. 3. Evaluation of the inorganic pyrophosphate effect on an OA marker and the Wnt pathway. Relative abundance of transcript for (**A**) IGFBP-3, (**B**) Wnt5a, (**C**) Wnt5b, (**D**) Ank, and (**E**) Enpp1 was normalized to RP29. The reported data are represented as mean ± SEM (n = 12 rats). Significance is indicated as * when compared to Control and # when compared to IL-1β ($p < 0.05$). Ank—inorganic pyrophosphate transport regulator; Enpp1—ectonucleotide pyrophosphatase/phosphodiesterase 1; IL—interleukin; IGFBP3—insuline like growth factor binding protein 3; OA—osteoarthritis; PPi—inorganic pyrophosphate; RP—ribosomal protein; SEM—standard error of the mean; Wnt—wingless-related integration site.

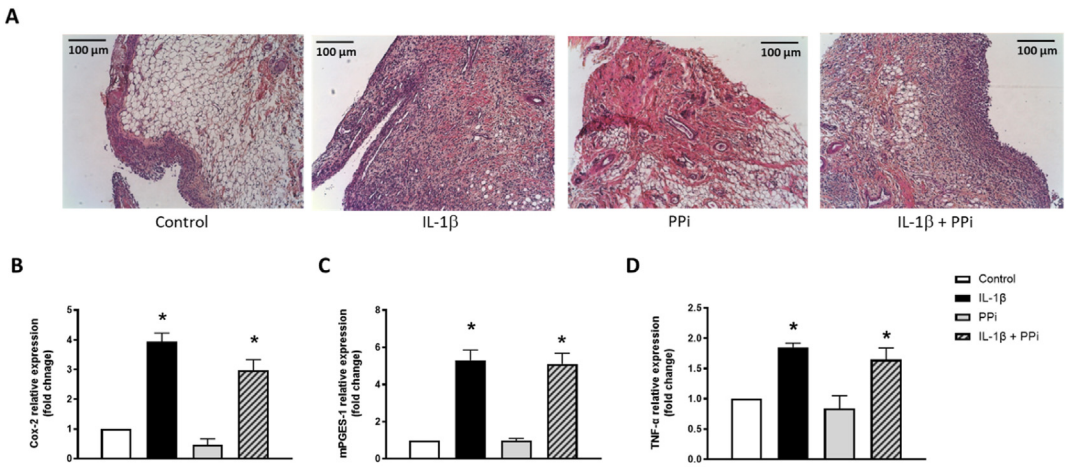

Fig. 4. Evaluation of the inorganic pyrophosphate effect on the synovial membrane inflammation in a rat model of knee cartilage damage induced by IL-1β. (**A**) Representative images of synovial membrane tissue sections highlighted by HES staining. The black scale bar corresponds to 100 µm. Total RNA was extracted from synovial membrane. The relative abundance of transcript for (**B**) Cox-2, (**C**) mPGES-1, and (**D**) TNF-α was normalized to RP29. The reported data are represented as mean ± SEM (n = 12 rats). Significance is indicated as * when compared to Control and # when compared to IL-1β ($p < 0.05$). Cox-2—cyclo-oxygenase type 2; HES—hematoxylin-eosin-saffron; IL—interleukin; mPGES-1—microsomal prostaglandin E_2 synthase-1; PPi—inorganic pyrophosphate; RP—ribosomal protein; SEM—standard error of the mean; TNF—tumor necrosis factor.

mobility. There is a crucial need to find suitable therapeutics that could protect cartilage to prevent its degeneration and OA symptoms [1,18]. The main findings of this article are that PPi improves IL-1β-induced cartilage damage in rat model of knee OA in vivo but not the associated inflammation of synovial membrane. These results suggest that PPi could be a molecule of interest to restrict OA progression.

The pro-inflammatory and pro-catabolic cytokine IL-1β is involved in OA progression by promoting joint tissue inflammation, chondrocyte dedifferentiation, and ECM degradation [19,24,25]. The IAI of IL-1β in rat knee joints has been utilized to quickly induce cartilage degradation and synovial inflammation similar to that seen in people with OA [38]. The decrease of PPi transport has been shown to induce dedifferentiation in a Wnt5a-dependent manner in a rat in vitro OA model with chondrocytes exposed to IL-1β. The ePPi addition participated in reversing this adverse situation, making ePPi a potential therapeutic agent to overcome the deleterious activation of Wnt5a and protect cartilage [28,29]. Thus, the aim of our study was to investigate if this PPi protective effect could be transposed in vivo. To do so, a single IAI of PPi was evaluated in a rat model of knee cartilage damage induced by IL-1β.

When used alone and at a concentration of 0.1 mM in a healthy knee joint, PPi showed no significant difference compared to the "Control" condition except for some cartilage ECM components with an increase of PGs (total sulfated GAGs and versican) and collagens (improved network and Col2). In addition, it halved the expression of the OA marker IGFBP-3. Surprisingly, PPi alone increased the expression of the hyaline marker Col2 and the dedifferentiation marker versican at the same time. At our lab, we observed that PPi used alone at 1 mM increased cartilage catabolism and bone fragility (data not shown). This adverse effect on bone can be explained by the fact that PPi is an inhibitor of mineralization [40–42].

The PPi effect was investigated in vivo with a single IAI of 0.1 mM in a rat model of knee cartilage damage induced by IL-1β. We demonstrated that PPi had not influence on the inflammation induced by IL-1β in the synovial membrane. Although PPi did not improve synovial inflammation, it showed remarkable results to protect cartilage from IL-1β induced degeneration. PPi had a positive effect to counteract cartilage damage with features similar to OA by improving cartilage cracks and ECM loss. It reduced IL-1β deleterious impact to prevent IGFBP-3 expression, chondrocyte dedifferentiation, and ECM catabolism. Wnt5a is a very important factor in cartilage homeostasis, so its increased expression will induce a profound change in the anabolic/catabolic balance of cartilage [43–45], but also in the subchondral bone cells [46,47]. The PPi protectiveness seems to hinder Wnt5a pathway, which is a major player in chondrocyte response to IL-1β and OA progression [44,45], through Enpp1 rather than Ank. Shi et al. previously confirmed that the silencing of Wnt5a protects from IL-1β-induced ECM degradation [48]. Moreover, Ding et al. showed that synthetic analogues of PPi, also called bisphosphonates, are able to alleviate OA by targeting Wnt5a [49]. Recently IGFBP-3 has also been described to play an important role in the onset of OA [50,51]. Consequently, PPi could be capable of repressing OA establishment by targeting Wnt5a and IGFBP-3 via a mechanism which remains to be deciphered [52].

The perspectives of this work would be to examine if the PPi protective capacity could be transferred to an in vivo OA model with cartilage damage other than an IL-1β-induced one [38] to check if this capacity is IL-1β-dependent or not. Among the various existing in vivo models, it can be found enzymatic and chemical approaches with respectively IAI of collagenase [12] and mono-iodoacetate [53], surgery with destabilization of the medial meniscus [54], and spontaneous OA depending on genetic background [55]. It would also be interesting to test a large-scale animal model and a route other than IAI to evaluate PPi biodistribution and efficacy to the joint (e.g., by mouth). An improvement of the PPi formulation could enhance and extend its beneficial effects on cartilage, for example with vectorization by using nanoliposomes [56,57] or hydrogel bioengineering [58–60]. Even if the results we obtained with rat are promising to use PPi to alleviate OA, experiments with human samples have to be performed in vitro with healthy/OA human chondrocytes and ex vivo (cartilage explants). Those experiments are required to ensure PPi treatment could be clinically transferable. If PPi stability is in question, another option would be to develop the application of bisphosphonates that are non-metabolizable PPi analogues [49,61]. To sum up, our results demonstrated that PPi could be used as a potential treatment to prevent cartilage degeneration and impair OA establishment.

Author contributions

Conception of the study, AB; design of the study, MK and AB; acquisition of data, MG, MK and AB; analysis and interpretation of data, ÉV and AB; writing—original draft preparation, ÉV and AB; writing—review and editing, ÉV and AB; supervision and project administration, AB; funding acquisition, AB. All authors have read and agreed to the published version of the manuscript.

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Ethics approval of research on animals

The animal study protocol was approved by the local ethics committee for animal experimentation of Université de Lorraine (protocol code: APAFIS#6624-2016042215241254 and date of approval: 10/10/2016) for studies involving animals. Discomfort and welfare were evaluated daily by the animal caretakers. Rats were fed a standard diet and had access to tap water ad libitum. European ethical guidelines for the care and use of laboratory animals have been respected throughout the study period.

Declaration of competing interest

The authors declare no conflict of interest.

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