Original Article

Platelet-rich plasma combined with ozone prevents cartilage destruction and improves weight-bearing asymmetry in a surgery-induced osteoarthritis rabbit model

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Background: Osteoarthritis (OA) is the most common degenerative disease in older adults and its treatment remains unsatisfactory. This study aimed to evaluate the effectiveness and explore the therapeutic mechanisms of the combination of platelet-rich plasma (PRP) and ozone (O_3) for knee OA.

Methods: Thirty male rabbits were randomly divided into five groups (Control group, OA group, PRP group, O_3 group, and PRP + O_3 group). Rabbit model of OA were induced by improved Hulth surgery. Gross articular observation, histopathological examination and cartilage scoring system were used to assess the articular cartilage destruction. The bone morphogenetic protein-2 (BMP-2) mRNA expression in joint fluid was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The expression of type II collagen, matrix metalloproteinase-1 (MMP-1) of cartilage was detected via Immunohistochemistry. Pain behavior was observed by percent ipsilateral weight-bearing (PIW) asymmetry.

Results: The content of platelet in PRP was increased by 6.2-times that in whole blood. Among induced OA groups (the OA, PRP, O_3 and PRP + O_3 group), PRP + O_3 significantly inhibited the surgically induced increase in gross articular alterations, histopathological damage of cartilage and Mankin score when compared to the OA, PRP and O_3 groups ($P<0.05$). Observed pain behavior by weight-bearing asymmetry, in the PRP + O_3 group was reversed at 3 and 6 weeks after the administration of PRP + O_3 (PIW asymmetry: –10.66%±1.172%). In addition, surgery-induced BMP-2 mRNA expression was significantly downregulated after the treatment of PRP, O_3 and PRP + O_3 ($P<0.01$). PRP + O_3 group significantly increased the expression of type II collagen but decreased MMP-1 of cartilage in comparison to OA, PRP and O_3 groups ($P<0.05$) by immunohistochemical analysis.

Conclusions: PRP combined with O_3 may prevent cartilage destruction and improve weight-bearing asymmetry by restoring homeostasis between anabolism and catabolism of extracellular matrix in progressive OA. Furthermore, a combination of PRP and O_3 might achieve even better results than the two agents alone.

Keywords: Platelet-rich plasma (PRP); ozone (O_3); osteoarthritis (OA); cartilage

Submitted Jun 06, 2021. Accepted for publication Sep 29, 2021.
doi: 10.21037/apm-21-1510
View this article at: https://dx.doi.org/10.21037/apm-21-1510

Introduction

Osteoarthritis (OA), a common disease in the elderly, is a degenerative condition producing functional disorders due to structural alterations affecting the entire joint. Impaired homeostasis of the articular cartilage plays a critical role in OA progression (1). Currently, treatments to halt the initiation and progression of OA are unsatisfactory (2). Thus, it is essential to develop new strategies to treat this disease.

Platelet-rich plasma (PRP) is centrifuged from whole blood, and is known to produce highly concentrated platelets and associated signaling molecules, including growth factors, chemokines and other cytokines. Several studies have shown that PRP holds potential to stimulate cartilage repair and delay joint arthroplasty in the management of knee OA (3-5). PRP is the subject of widespread experimental and clinical research, since its optimal method of administration, centrifugation protocols, and combination with other close agents has not been established (6-9). Intra-articular administration of ozone (O_3) dissolves in synovial fluid, where it oxidates antioxidants and polyunsaturated fatty acids with the generation of reactive oxygen species and lipid oxidations (10). O_3 has been investigated for its capacity to resist progression of OA based on its direct and indirect free radical scavenging ability to normalize the cellular redox balance. It produces articular cartilage protective effects by inhibition of matrix metalloproteinase (MMP), and regulation of inflammation, which damages the cartilage matrix through the actions of cytokines (11,12). Our previous research has suggested that O_3 alone may improve cartilage repair and functional joint recovery, as well as demonstrate efficacy in combination with various injectional agents (13,14). It has been shown that the association of PRP and O_3 can ameliorate oxidative stress, improve recovery and alleviate pain and functional disorders, making it a suitable option in treating knee OA (15,16). However, the pathological evidence for the efficacy of PRP combined with O_3 in the treatment of OA remains unclear.

This study aimed to provide first evidence on pathology, pain-like behavior, and extracellular matrix metabolism of the efficacy of PRP combined with O_3 on a surgery-induced rabbit model, clarifying the theoretical basis for the administration of the combination of PRP and O_3 in OA knees.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://apm.amegroups.com/article/view/10.21037/apm-21-1510/rc).

Methods

PRP and O_3 preparation

Autologous blood samples were extracted to prepare PRP following a double-centrifugation protocol reported by Landesberg *et al.* (17). Briefly, after rabbits were deeply anesthetized intravenously with pentobarbital (30 mg/kg) through the ear vein; then 9 mL of whole blood samples were withdrawn from the central ear artery and transferred into 10 mL vacutainers containing 1 mL of 3.8% sodium citrate as anticoagulant. An aliquot of 0.2 mL of each blood sample was drawn out for platelet counting, and the blood samples were centrifuged at 200 g for 10 min. After the first centrifugation, the plasma and the buffy coat layer were centrifuged at 200 g for another 10 min in a new centrifuge tube. Subsequently, platelet-poor plasma on the 3/4 upper of the centrifuge tube was removed, and the remaining liquid was shaken evenly to obtain PRP. Another 0.2 mL PRP was drawn out for platelet counting. Platelet and leukocyte counts in whole blood and PRP were examined with a hematology analyzer.

O_3 was generated at 20 μg/mL by using OZOMED medical O_3 therapeutic apparatus (Xkyr Tech, Beijing, China).

Animals and experimental design

This study complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85-23, revised 1996) and the Animal Management Regulation of the Chinese Ministry of Health, and was approved by the institutional ethics committee of First Hospital of Tsinghua University (RECLA20-01). Thirty male New Zealand rabbits (mean weight: 2.4 kg) were obtained from the Beijing Fangyuanyuan Experimental Animal Co., Ltd. (Medical Experimental Animal Number: SCXK2014-0012). All rabbits were allowed free access to food and water under a temperature-controlled environment with regular day-night cycles. Efforts were made to limit the number of animals used and minimize suffering. Humane endpoints were set; during the experiment, when animals that suffered from chronic or severe pain or distress would be humane euthanized.

All rabbits survived to the end of treatments period. According to the results of drawing lots with playing cards, thirty rabbits were randomly divided into five groups

Table 1 Primer sequences for RT-PCR

Genes	Primer sequences	Fragment size (bp)
BMP-2	Forward 5'-GGTTTGTGGTGGAAGTGACC-3'	88
	Reverse 5'-TCATCTGGGTGCAAAGACCT-3'	
GAPDH	Forward 5'-AGACACGATGGTGAAGGTCG-3'	164
	Reverse 5'-TGCCGTGGGTGGAATCATAC-3'	

RT-PCR, reverse transcription polymerase chain reaction; BMP-2, bone morphogenetic protein-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(n=6 each group): Control group (without treatment), OA group (OA model), PRP group (OA induced rabbits were injected with 0.6 mL PRP), O_3 group (OA induced rabbits were injected with 40 μg O_3), and PRP + O_3 group (OA induced rabbits were injected with 0.6 mL PRP and 40 μg O_3). Confounders were not controlled. The sample size and dosages used herein were determined from previous experiments, in which PRP or O_3 was intra-articular administered into OA rabbits (14,18-21). No criteria for including and excluding were set and no animals and data points were excluded. Due to the nature of the treatment, only therapists aware of the group allocation to ensure the correct intervention is provided; assessors and researchers were blinded to the group allocation.

OA was surgically-induced in the left knee of experimental rabbits by the improved Hulth method, according to previous reports (22). Specifically, the key procedure of the improved Hulth method involves anterior cruciate ligament and medial collateral ligament transections, and meniscectomy. Six weeks after surgery, PRP, O_3, and PRP combined with O_3 were injected into the left knee of rabbits once a week for 6 weeks.

Analysis of weight-bearing

Between the left (ipsilateral) and right (contralateral) hindlimbs, development of the asymmetry in static weight-bearing distribution was utilized as an index of joint pain-like symptoms, represented by a decrease in percent ipsilateral weight-bearing (PIW), which was calculated as weight on the ipsilateral hindlimb divided by weight on bilateral hindlimbs and multiplied by 100%. Static weight-bearing was assessed at 0, 6, 9 and 12 weeks post-surgery using YLS-11A Incapacitance Tester (Zsdc Tech, Beijing, China). In the tester, the average weight on each hindlimb over 5 sec was recorded for five trials by transducers, when rabbits were habituated to a relatively static position. The average PIW per rabbit at each time point was used in the statistical analyses.

Measurement of the joint fluid BMP-2 mRNA level

One week after the last injection, the joint cavity was flushed with 1 mL normal saline 3 times, and this fluid from the joint cavity was collected for detection of bone morphogenetic protein-2 (BMP-2) relative expression via quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis method. Total RNA in joint fluid was extracted using TRIzol Reagent (Invitrogen, CA, USA) and reverse transcribed into complementary DNA (cDNA). Relative expression of BMP-2 was determined by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression level, and was calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences and product lengths are listed in *Table 1* internal reference.

Histological analysis

One week after the last injection, the rabbits were sacrificed with overdose of pentobarbital (120 mg/kg) and left knee joints were dissected. The joint samples of the articular cartilage were stored in 4% paraformaldehyde for 7 days, and decalcified in 10% ethylene diamine tetraacetic acid (EDTA) solution for 3 months, then dehydrated by ethanol solution. Next, the samples were embedded in paraffin and cut into sections and stained with hematoxylin and eosin (H&E) or toluidine blue (TB) for histological assessment according to the Mankin scoring system, including the cartilage surface lesion, structural compromise, loss of matrix staining, loss of tidemark integrity and proportions of the lesion site (23).

Immunohistochemical analysis

Immunoreactivity of type II collagen and MMP-1 was

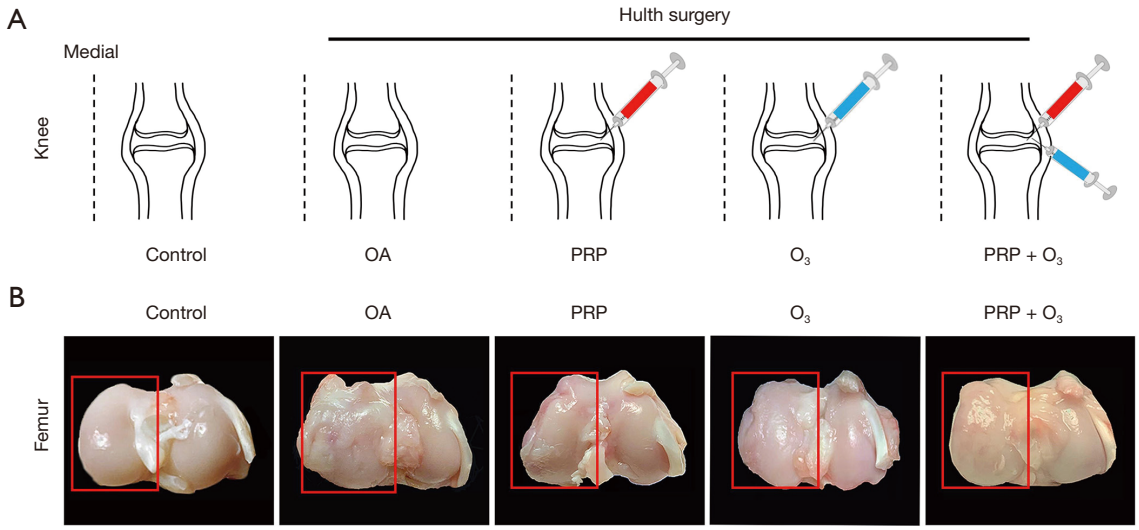

Figure 1 Design of intra-articular injection and comparisons of gross appearance of femoral articular surface. (A) Sketches of left knee show the scheme of intra-articular injection. (B) Representative gross images of osteoarthritic severity of articular surface (femur) 6 weeks post-treatment. Red frames indicate the area of OA inspection. The magnification of the image is ×2. All groups (n=6). OA, osteoarthritis; PRP, platelet-rich plasma; O_3, ozone.

assessed. Briefly, endogenous peroxidase activity was blocked by 0.3% H_2O_2 for 30 min, followed by rinsing with PBS and blocking with bovine serum albumin V blocking solution for 30 min. Sections were incubated overnight at 4 ℃ with antibodies against type II collagen (1:200, Proteintech, CA, USA) or MMP-1 (1:100, Proteintech, CA, USA). After rinsing with PBS, sections were incubated with goat anti-rabbit IgG and developed using DAB color reagent. The presence of antigen in the cartilage was estimated by calculating the number of chondrocytes with positive staining. The number of total chondrocytes and positively stained chondrocytes in three central regions of articular cartilage were counted using Image Pro Plus version 5.1 software. The percentage of antigen staining positive cells in different group were then determined.

Outcomes

Primary outcome was the degree of cartilage alterations and destruction. Secondary outcomes were the improvement of weight-bearing asymmetry and metabolism in extracellular matrix.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). The SPSS 21.0 (International Business Machines Corporation, NY, USA) software and GraphPad Prism 8.0 (GraphPad Software, Inc., CA, USA) software were used for statistical analysis. Normal distribution of the data was tested using the D'Agostino and Pearson normality test. If data did not present normal distributions, then nonparametric analyses were used.

Differences in PIW asymmetry over time were assessed by two-way analysis of variance (ANOVA), followed by Bonferroni post-tests for comparison between more than two groups. One-way ANOVA with Tukey post-test was used for other data evaluations between multiple groups. Results were considered statistically significant when $P<0.05$.

Results

Assessment of PRP

The Hulth-induced OA rabbits were treated with PRP, O_3, or PRP + O_3 according to the intra-articular strategy shown in *Figure 1A*. In blood samples of PRP and PRP + O_3 groups, the average concentration of platelet in whole blood and PRP was $(346.25\pm63.57)\times10^9$/L and $(2,151.75\pm439.25)\times10^9$/L, respectively. The platelet density was 6.2-fold in PRP of that in the peripheral blood, showing significant difference ($P<0.05$). In contrast, the leukocyte counts decreased in PRP relative to whole blood. For whole blood, the

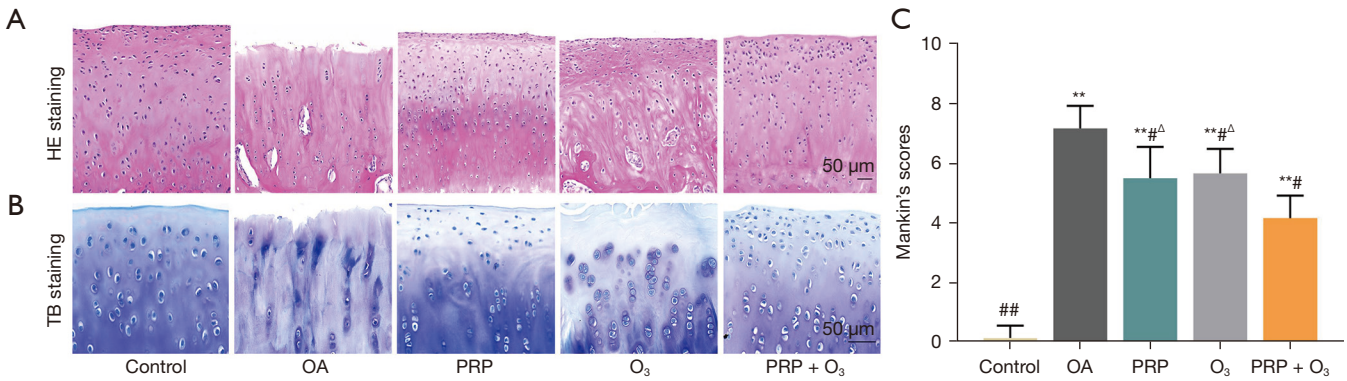

Figure 2 Histological images and analysis of the articular surface (femur) cartilages. (A) H&E and (B) TB stained sections of articular cartilage. Scale bar: 50 μm. (C) Cartilage destruction evaluated with the Mankin scoring system. All groups (n=6). The groups compared with Control are indicated if significantly different as **P<0.01; and compared with OA as #P<0.05, ##P<0.01; and compared with PRP + O_3 as ΔP<0.05. H&E, hematoxylin and eosin; TB, toluidine blue; OA, osteoarthritis; PRP, platelet-rich plasma; O_3, ozone.

mean leukocyte count was (9.84±1.14)×10^9/L, while for PRP, the mean leukocyte count was (2.39±0.92)×10^9/L. These data indicated that the prepared PRP was not rich in leukocyte.

PRP combined with O_3 alleviates gross articular alterations of OA rabbits

Gross articular observation of the OA group showed the loss of anterior cruciate ligaments and medial meniscus, with marked changes in OA, including cartilage erosion, osteophyte formation, and synovial edema and thickening around the femur (*Figure 1B*). The visual observation was consistent with OA characteristics (24), indicating that OA was successfully induced. However, significantly decreased cartilage defect and synovial thickness were observed in PRP, O_3, and PRP combined with O_3 treated rabbits.

PRP combined with O_3 prevents cartilage destruction of OA rabbits

In OA group rabbits, H&E and TB staining showed marked increases in surface cartilage damage, disordered arrangement of chondrocytes, and unevenly stained extracellular matrix, compared with Control group rabbits (*Figure 2A,2B*). In contrast, treatment of PRP, O_3 and PRP + O_3 groups resulted in less surface loss and chondrocyte disorder in articular cartilage compared with the OA group. Meanwhile, clear evenly stained extracellular matrix was observed in the PRP + O_3 group compared with the PRP and O_3 group. Articular Mankin score (*Figure 2C*) of the OA group was significantly increased compared with the Control group (P<0.01). While both PRP or O_3 exposure caused a steady reduction in Mankin scoring (both P<0.05), the combination of PRP + O_3 revealed a higher protective effect when compared to either single agent (P<0.05).

PRP combined with O_3 improves weight-bearing asymmetry of OA rabbits

Progression of pain-like symptoms following the improved Hulth surgery were detected by the weight-bearing analysis (*Table 2*). At baseline, the PIW of all experimental rabbits was close to 50%. Quantitative data showed that all the OA induced rabbits (OA group, PRP group, O_3 group and PRP + O_3 group) had significantly lower PIW at each post-surgery time point (6, 9, 12 weeks post-surgery) when compared to the baseline (P<0.05). Six weeks after surgery, before the first time of treatment, there was no significantly difference in PIW between OA induced rabbits. At 9 weeks after the surgery, the O_3 and PRP + O_3 groups had significantly better weight-bearing scores than the PRP group (P<0.05, P<0.01). After 12 weeks, the PRP + O_3 group had better PIW than the O_3 group (P<0.01, P<0.05). The effect of PRP combined with O_3 reducing static weight-bearing asymmetry of OA rabbits showed earlier and more obvious, compared with single treatments.

PRP combined with O_3 regulates extracellular matrix-related genes of OA rabbits

The BMP-2 is closely related to the anabolism of the

Table 2 Comparison of effect on PIW asymmetry (%, n=6, $\bar{x}$±s)

Group	Baseline (before surgery)	Six weeks post-surgery	Nine weeks post-surgery	Twelve weeks post-surgery
Control	49.66±1.40	50.945±1.39	49.54±1.38	49.12±1.36
OA	49.76±1.57	36.12±1.62*	37.71±1.70*	38.35±1.93*
PRP	50.90±1.47	36.32±1.69*	39.64±1.74*	44.57±1.62*##
O_3	50.84±1.29	37.91±1.71*	41.24±1.51*#	42.71±1.53*#
PRP + O_3	49.98±1.51	34.88±1.66*	42.11±1.76*##	45.12±1.69*##

The groups compared with baseline are indicated if significantly different as *P<0.01; and compared with 6 weeks post-surgery as #P<0.05, ##P<0.01. PIW, percent ipsilateral weight-bearing; OA, osteoarthritis; PRP, platelet-rich plasma; O_3, ozone.

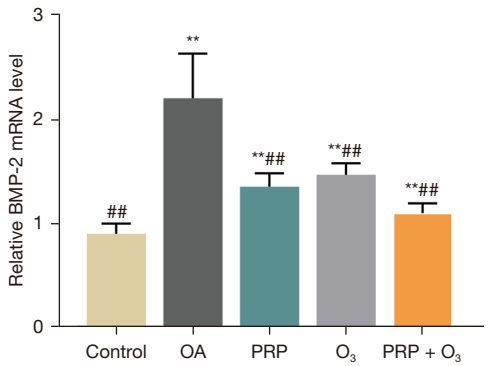

Figure 3 The mRNA expression levels of the BMP-2 in the joint fluid were detected by qRT-PCR. GAPDH was used as the internal control. All groups (n=6). The groups compared with Control are indicated if significantly different as **P<0.01; and compared with OA as ##P<0.01. BMP-2, bone morphogenetic protein-2; qRT-PCR, quantitative reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OA, osteoarthritis; PRP, platelet-rich plasma; O_3, ozone.

extracellular matrix and osteophyte formation. As shown in *Figure 3*, relative mRNA expression of BMP-2 in the joint fluid of OA group rabbits was higher than that of Control group rabbits (P<0.01). Compared with the OA group, expression of BMP-2 mRNA in PRP, O_3 and PRP + O_3 groups were decreased (P<0.01, P<0.01, P<0.01).

The immunohistochemical experiments were carried out to evaluate the catabolic condition of extracellular matrix in cartilage of OA rabbits (*Figure 4A,4B*). Surgically-induced decrease of type II collagen in cartilage could be largely reversed by treatment of PRP and/or O_3, while the increased expression of MMP-1 in OA model was dramatically down-regulated after intra-articular treatment. Notably, the improvement effect of PRP + O_3 was more obvious than that of PRP or O_3 alone (P<0.05).

Discussion

Our findings demonstrate a promising effect of the combination of exposure to PRP and O_3 on the progression of surgically-induced OA. The results show the effect on articular damage prevention of this combination and enhanced percent static weight-bearing of the ipsilateral hindlimb after the administration with PRP + O_3. Lastly, we showed that the administration with PRP + O_3 reduced synovial BMP-2 mRNA expression levels and numbers of cartilage type II collagen and MMP-1-immunolabeled chondrocytes. These findings represent reliable evidence that PRP combined with O_3 regulates the homeostasis of extracellular matrix metabolism, and subsequently prevented the cartilage destruction and improved weight-bearing asymmetry of OA in rabbits. Notably, our current research includes direct pathological evidence on the appearance of cartilage in controls as well as after combination of treatments.

OA is a debilitating disease that reduced life quality and leads to permanent disability in aged populations. One simple pharmacological agent is unable to address both symptoms and pathology in the clinical situation of OA (2). Therefore, seeking potentially multimodal treatments which have complementary mechanisms is needed in OA management. PRP is thought to conduct short-term clinical improvements by having an influence on the entire joint environment of cartilage degenerative progression. Clinical outcomes might be influenced by methodological variables, which need to be studied to optimize intra-articular PRP treatment in OA progression. Recently, Dernek *et al.* showed that patients receiving PRP treatment combined with O_3 achieve earlier alleviation in OA symptoms, when compared with PRP treatment alone (15). However, there are a few limitations to this study, due to its retrospective nature and lack of histopathological examination to

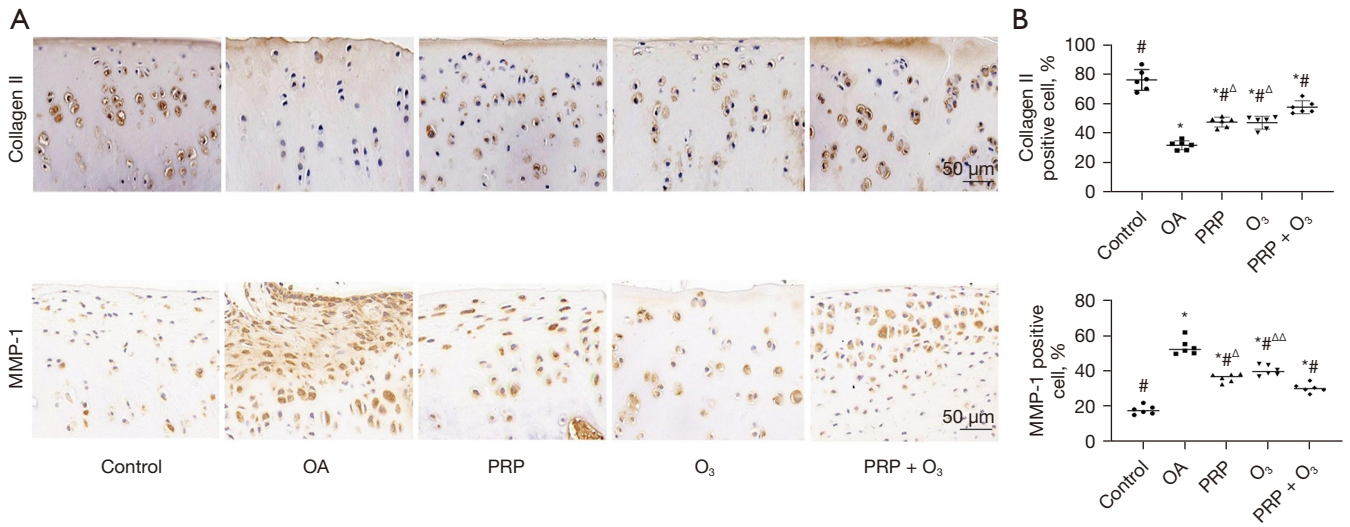

Figure 4 (A,B) Immunohistochemistry analysis of type II collagen and MMP-1 in sections of femoral articular surface. Scale bar: 50 μm. All groups (n=6). The groups compared with Control are indicated if significantly different as *P<0.05; and compared with OA as $^{\#}$P<0.05; and compared with PRP + O_3 as $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01. MMP-1, matrix metalloproteinase-1; OA, osteoarthritis; PRP, platelet-rich plasma; O_3, ozone.

demonstrate improvement of the articular cartilage.

There is a growing realization that the impaired homeostasis of articular cartilage contributes to OA pathogenesis and progression (25,26). Indeed, many cytokines correlate with articular components degradation, which exceed the capacity of restoration and leads to extracellular matrix degradation in OA joints. BMP-2 is one of the key factors involved in differentiation belonging to the TGF-β superfamily and shows important effects in all phases of chondrogenesis, including the synthesis of type II collagen and regulation of chondrocytes anabolism (27). Whereas BMP-2 has potent anabolic actions, it can also lead to the degradation of aggrecan and boost OA-like cartilage loss (28). Levels of BMP-2 are found elevated in varied OA cartilage layers and osteophytes according to the extent of OA (29-31). In addition, stimulation of pro-inflammatory cytokines IL-1β and TNF-α can increase the expression of active BMP-2 in chondrocytes (30). Research indicated that the presence of chronic low-grade inflammation in articular cartilage contributes to the development of OA (32,33). All of these suggest that BMP-2 plays a critical role in the pathogenesis progress of OA. Recent studies revealed that joint fluid BMP-2 concentration has a significant correlation with the radiographic and symptomatic severity of joint damage in knee OA (34,35). Up-regulated BMP-2 level in a post-traumatic joint can contribute to tissue repair by promoting matrix synthesis, while on the other hand leading to a higher level of degeneration in collagen by stimulating MMPs expression. It is reported that the MMP family plays a primary role in progressive OA, through a critical role in the degradative regulation of cartilage matrix components (36). MMP-1 is a rate-limiting type II collagenase causing degradation and destruction of extracellular matrix and activates other MMP family members to play an essential role in OA (37). Consistent with the results of previous studies, the Hulth-induced OA rabbits showed elevated expression of BMP-2 mRNA in joint fluid, and MMP-1 expression in the articular cartilage. Moreover, histopathological alterations included destruction and calcification of cartilage matrix in our OA model. Briefly, these findings suggest a strong correlation between the synovial expression of BMP-2 mRNA and degradation of extracellular matrix in OA progression.

Several studies have confirmed that PRP concentrates a variety of platelets, growth factors and bioactive components, which offer a suitable microenvironment for cell proliferation and matrix accumulation. In our study, the timing of inhibition of PRP combined with O_3, and its effects are clarified. As a direct and indirect oxidant for cartilage repair, O_3 could generate reactive oxygen products and lipid oxidation species within the joint. Recent studies have suggested that oxidized lipid species may stimulate

platelets activation, ensuring that the coagulation cascade is triggered during fibrin retraction and fibrinolysis, perhaps improving efficacy by confining PRP to the articular cavity (3,38,39). We hypothesize that PRP combined with O_3 may coordinate the release of signaling elements, causing PRP to better adhere to the cartilage surface and synovium, and inhibit the release of MMPs that degrade the extracellular matrix in knee OA. These may explain the better therapeutic effect of PRP combined with O_3.

In knee OA, pain is the prominent symptom and is the dominant driver of clinical decision making (1). The distribution of weight-bearing analysis is of great importance in the evaluation of OA-induced pain, and ipsilateral lower limb weakness is often observed in patients with knee OA (40). Our previous studies suggested that intra-articular O_3 injection improved the static weight-bearing asymmetry of OA rabbits (14). Similarly, Gowler *et al.* used weight-bearing asymmetry to evaluate analgesic effect with a surgically-induced model of OA in animals (41). In the present study, the combination of PRP and O_3 synergistically facilitated cartilage protection and catabolism reduction and thus eventually augmented the ipsilateral weight-bearing recovery, in line with previous studies that showed that articular homeostasis contributed to OA analgesic action.

The Hulth-induced model, resulting in joint instability, produces a traumatic form of OA and reliably mimics the pathogenesis and pathological alterations of human OA (42). In this study, the main limitation is the absence of Control groups administered with different doses, primarily because of the limited number of experimental animals. Although additional studies to identify optimal PRP preparation protocols and dosage regimens and are required, these results confirm the effectiveness of the combination of PRP and O_3 to reverse the extracellular matrix catabolism associated with OA and subsequently establish the ability of the treatment to protect articular cartilage and ameliorate changed weight-bearing behaviors due to OA progression.

Conclusions

In this animal study, we evaluated the combination of intra-articular injection of PRP and O_3 as a promising strategy for the treatment of knee OA, and which may be superior to PRP or O_3 alone. In addition, the protective effect of PRP combined with O_3 may be involved in the metabolic homeostasis regulation of extracellular matrix.

Acknowledgments

The authors thank especially professor Daniel Porter and Jun Jiang for their insightful comments and assistance with language revision.
Funding: None.

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://apm.amegroups.com/article/view/10.21037/apm-21-1510/rc

Data Sharing Statement: Available at https://apm.amegroups.com/article/view/10.21037/apm-21-1510/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://apm.amegroups.com/article/view/10.21037/apm-21-1510/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. RECLA20-01) granted by the institutional ethics committee of First Hospital of Tsinghua University, in compliance with National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85-23, revised 1996) and the Animal Management Regulation of the Chinese Ministry of Health. A protocol was prepared before the study without registration.

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Cite this article as: Huang P, Wang R, Pang X, Yang Y, Guan Y, Zhang D. Platelet-rich plasma combined with ozone prevents cartilage destruction and improves weight-bearing asymmetry in a surgery-induced osteoarthritis rabbit model. Ann Palliat Med 2022;11(2):442-451. doi: 10.21037/apm-21-1510

