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Hyaluronic Acid Prevent Further Cartilage Damage of Osteoarthritis Based on Expression of Collagen Type II and Collagen Type X

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Abstract

BACKGROUND: Osteoarthritis (OA) is a degenerative joint disease characterized by changes in the structure of the subchondral articular cartilage. Chondrocytes are responsible for the synthesis and integrity of the extracellular matrix of articular cartilage. Hyaluronic acid (HA) is believed to have a potential protective effect on joint cartilage through chondroprotective.

AIM: This study compares the effect of HA administration on the expression of type-II collagen and type-X collagen in chondrocytes cartilage of end-stage OA. We hypothesized that there was an increase in the expression of COL2 and a decrease in COL10 in knee cartilage chondrocytes in end-grade OA patients with controls on HA.

MATERIALS AND METHODS: This study is experimental research (pre-and posttest control group design) with 20 samples divided into five groups, each consisting of four samples. Four different dosages of HA have been given to the treatment group: 0.1 mg/ml, 1 mg/ml, 2 mg/ml, and 3 mg/ml. Subsequently, collagen type II (COL2) and type X (COL10) were examined using the enzyme-linked immunosorbent assay method, and data were analyzed with SPSS 20.0

RESULTS: Our study revealed that COL2 expression was not significantly different between the control group and 0.1 mg/ml. Interestingly, with 1 mg/ml of HA, there was a markedly significant increase in the expression of COL2 ($p < 0.05$), and a further increase in dosage did not give an incremental effect. Conversely, the treatment of HA significantly suppressed the expression of COL10, but no enhanced suppression was found with increasing dose.

CONCLUSION: The administration of HA results in an increased number of COL2 and reduced number of COL10 and has the potential function of inhibiting the degeneration process in joint cartilage.

Introduction

Osteoarthritis (OA) is the highest prevalence chronic joint disease and a major source of pain and disability in the United States and worldwide. With increasing life expectancy and obesity rates worldwide, the risk of OA has also increased and has become one of the major problems in the global health system [1]. OA is a chronic disease of the joints that causes pain due to inflammation. OA usually occurs in the knee, hip, and spine joints because they are the joints that support body weight [2]. The global prevalence of radiograph confirmed Kellgren-Lawrence grade 2–4 knee OA was estimated to be 3.8% of the population (4.8% women and 2.8% men). Whereas in Indonesia, the prevalence of knee OA is relatively high, reaching 15.5% in the male population and 12.7% in the female population with a distribution of 5% at <40 years old, 30% at 40–60 years old, and 65% at >61 years old [3]. OA is a complex disease caused by various factors involving changes in the subchondral articular bone and cartilage structure. This condition leads to signs and symptoms associated with impaired cartilage integrity and subchondral bone changes. Changes in cartilage structure include changes in chondrocytes as well as extracellular matrix. The conversion of chondrocytes to hypertrophic-like chondrocytes makes the chondrocytes have a round shape with a larger size, expresses type-X collagen from the COL10 gene, decreased collagen type II (COL2) expression from the COL2A1 gene, and expresses several other markers such as matrix metalloproteinase 13, RUNX2, and osteocalcin. Hypertrophic-like chondrocytes stimulate damage to surrounding cells, especially in conditions of OA [4]. OA management aims to control pain, optimize joint function, reduce physical limitations, improve quality of life, and inhibit disease progression. Nonoperative treatment is an option to reduce symptoms of OA, one of which is by administering an intraarticular injection of Hyaluronic Acid [5], [6].

Intraarticular injection of HA has been shown to have a protective effect on joint cartilage through several mechanisms, including mechanical effects,

chondroprotective effects by reducing apoptosis and increasing chondrocyte proliferation, anti-inflammatory effects, and increased proteoglycan and glycosaminoglycan synthesis [7].

In normal cartilage, chondrocytes are responsible for the synthesis and integrity of the extracellular matrix of joint cartilage. Aggrecan (ACAN) and COL2 are major macromolecules and essential components in structuring the extracellular matrix. Therefore, damage in COL2 and loss of extracellular matrix components results in decreased matrix synthesis and joint cartilage tissue degradation regulation. Therefore, the balance between HA and COL2 is a critical parameter for determining the integrity of the extracellular matrix of joint cartilage [8]. In OA, articular chondrocytes differentiate into hypertrophic-like chondrocytes. The specific marker for hypertrophic-like chondrocytes is type-X collagen, expressed higher in patients with more severe cartilage degradation [4]. This study compares the effect of HA administration on the expression of type-II collagen and type-X collagen in chondrocytes cartilage of end-stage OA. We hypothesized that there was an increase in the expression of COL2 and a decrease in COL10 in knee cartilage chondrocytes in end-grade OA patients with controls on HA.

Methods

Design study

The research design is in vitro true experimental research conducted in a laboratory to treat the research sample's tissue. This study design uses posttest only on the control and treatment groups.

Participants

The population of the research subjects is patients with knee OA who will undergo total knee replacement surgery at Saiful Anwar Hospital, Malang, Indonesia, in January–February 2021. The respondent is from the regular patient (nonpavilion) population.

Sample

We used 20 cartilage tissues from adult patients with knee OA who underwent total knee surgery at RSUD Dr. Saiful Anwar in January–February 2021 as samples in this research. The samples were divided into five groups consisting of one control group and four treatment groups with a total of 20 sample chondrocytes was taken from articular cartilage. This sample was obtained from repetition formula. The treatment groups received HA in various doses, namely 0.1 mg/mL, 1 mg/ml, 2 mg/ml, and 3 mg/ml, respectively. The cartilage tissue sample was then cultured and incubated for 3 days until the overall number of normal and hypertrophic-like chondrocyte cells increased. There were five experimental groups in this study, so the number of samples for each experimental group can be found using the formula below. These numbers were tested with a significance level of 0.05.

$$n \geq \frac{15}{p} + 1$$

Description

n: repetition
p: experimental group

$$n \geq \frac{15}{5} + 1$$

$$n \geq 3 + 1 \rightarrow n \geq 4$$

From this formula, if the number of treatments is 5, the number of repetitions needed for each treatment group is 4. Therefore, if we need five treatment groups, a minimum of chondrocyte cell groups from articular cartilage is 20 preparations required.

Data source/measurement

After the ethical application was approved, the research was carried out for 4 weeks, namely January–February 2021. Samples were taken when participants were on total knee replacement surgery. Surgery and sampling in this operation take 2–3 h from the completion of anesthesia. The selection itself takes 15–30 min during surgery in progress.

OA type

The samples in this study were end-stage OA with osteophytes to the central intraarticular areas and/or joint space narrowing up to >50% according to Ahlbäck stages 2–5 [9]. Another classification is the Kellgren-Lawrence classification stage 3–4, where there were progressive cartilage destruction, subchondral bone exposure, and the presence of a hard ivory-like area called eburnation on the weight-bearing area (medial/lateral condyle) [10], [11].

HA

HA used in this study was the Umarone® which was given to chondrocyte cultures in culture media with different doses for each treatment group [12].

Type X and type II collagen level measurement

Type X collagen is produced by hypertrophic-like chondrocytes, whereas type II collagen is produced

by chondrocyte cells. Therefore, after three days of culture, there was an increase in the overall number of normal and *hypertrophic-like chondrocytes*. Then, the cultures were taken for the examination using enzyme-linked immunosorbent assay (ELISA) with Human COL2 antibodies and Human COL10 ELISA-kit (Bioassay Technology Laboratory®). These measurements were carried out after the administration of HA.

The inclusion and exclusion criteria

The inclusion criteria were the cartilage obtained from OA patients on total knee replacement procedure, normal chondrocytes (cobblestone), and hypertrophic-like chondrocytes were found in the culture field, cartilage cultures were confluent after 3 days of cell incubation (overall chondrocyte count increased per field), cartilage culture plates are not contaminated (bacteria), and if it is not included in the inclusion criteria, then it is grouped into the exclusion criteria.

Variables

Independent Variables: HA applied to culture cell media. Dependent Variables: Expression of COL10 and COL2 in chondrocytes.

To prevent bias in this study, we have selected samples based on inclusion criteria and maintained the quality of the study by controlling the control variables consisting of sampling in the end-stage of OA, how to collect and store articular cartilage, and process chondrocyte cell culture according to the standard. The independent variable in this research was administering HA to cell culture media. The independent variable in this research was the expression of COL10 and COL2 in chondrocytes. The Ethical Committee of Medical Research Faculty of Medicine Universitas Brawijaya has approved all protocols, and all subsequent experiments were carried out according to the relevant guidelines and regulations.

Statistical methods

All the samples of this study were analyzed with statistical software, and no one of the samples is missing to be analyzed because all of them include in the inclusion criteria. Data obtained were analyzed using the Shapiro–Wilk normality test to check the data distribution. If data are normally distributed, then the one-way analysis of variance (ANOVA) and Tukey parametric tests are performed, whereas if data are not normally distributed, the Kruskal–Wallis and Mann–Whitney nonparametric tests are performed. One-way ANOVA has an error rate of 5% or a 95 % level of confidence, and the Kruskal–Wallis test was used to compare the difference of means among all groups. The *post hoc* test using Mann–Whitney and Tukey test was performed to assess all possible pair-wise comparisons between groups. The $p < 0.05$ was considered statistically significant. Data analysis was performed using SPSS version 25.0 software.

Results

Descriptive data

Descriptive analysis is intended to determine the general description of the research variables. In addition, this descriptive analysis is expected to provide an overview of the state of patient data. To find out the description for each variable can be seen in this table (Table 1).

Table 1: Descriptive results

Collagen	Dose	n	Mean	Std. Deviation
Collagen Type II	0	5	78.378	2.979
	0.1 mg/ml	5	74.044	8.086
	1 mg/ml	5	122.067	13.872
	2 mg/ml	5	105.845	7.440
	3 mg/ml	5	113.422	8.573
Collagen Type X	0	5	24.096	0.537
	0.1 mg/ml	5	4.793	0.256
	1 mg/ml	5	6.619	0.362
	2 mg/ml	5	6.182	0.451
	3 mg/ml	5	5.954	0.144

Based on the description above, we have not shown the results of the study. Therefore, a hypothesis test was conducted using a one-way ANOVA to find the research results with an error rate of 5% or a 95 % level of confidence. However, before using parametric statistics, the first test of normality and data homogeneity is required. The normality test using Shapiro–Wilk showed that the significance value (p) of COL2 is $p = 0.185$. In contrast, COL10 is $p < 0.0001$, so it can be concluded that COL2 distribution is normal while COL10 is not distributed normally.

Furthermore, for the homogeneity test (Levene Test), COL2 and COL10 have significance $p = 0.0067$ and $p = 0.435$ consecutively, so both results have homogeneous data (Table 2). Therefore, from the data, researchers will use the ANOVA test to be used on COL2 and nonparametric statistical testing on COL10. Furthermore, a one-way ANOVA test determines differences between treatment groups and tests whether there is a significant difference between one treatment and another. If $p < 0.05$ for COL2 parameter, hypothesis 0 (H_0) is rejected (Table 3). As a result, there were

Table 2: Normality and homogeneity test results

Test	Collagen type II	Collagen type X
Normality test		
N	25	25
P	0.185	0.000
Homogeneity test		
Levene Statistic	2.608	2.225
df1	4	4
df2	20	20
P	0.067	0.435

Table 3: One-way ANOVA, Kruskal–Wallis, and Spearman's Correlation Test Results

Test result	Parameter	F	p	r	Information
One-way ANOVA test	Collagen Type II	28.992	0.000	-	Significant
Kruskal–Wallis test	Collagen Type X	-	0.000	-	Significant
Spearman's correlation	Dose – Type II Collagen	-	0.000	0.665	Significant
test	Dose – Type X Collagen	-	0.101	-0.335	Nonsignificant

ANOVA: Analysis of variance.

meaningful differences between the treatment groups and obtained a significance p for COL2, p < 0.0001. Hence, it can be concluded that there were significant differences in influence between doses with a 95% confidence level.

Then, we conducted Tukey tests for COL2 to compare each treatment group. It was found that doses of 0 mg/ml and 0.1 mg/ml did not have significant differences because it has a p = 0.936, while doses of 0 mg/ml and 1 mg/ml had considerable significance with doses of 1 mg/ml, 2 mg/ml, and 3 mg/ml significance which has a p < 0.0001 (Table 4).

Table 4: Tukey test results for collagen type II

Dosage differences	Average difference	p	Information
0			
0.1 mg/ml	4.333	0.936	Nonsignificant
1 mg/ml	−43.689	0.000	Significant
2 mg/ml	−27.467	0.001	Significant
3 mg/ml	−35.044	0.000	Significant
0.1 mg/ml			
1 mg/ml	−48.022	0.000	Significant
2 mg/ml	−31.800	0.000	Significant
3 mg/ml	−39.378	0.000	Significant
1 mg/ml			
2 mg/ml	16.222	0.062	Nonsignificant
3 mg/ml	8.645	0.552	Nonsignificant
2 mg/ml			
3 mg/ml	−7.577	0.666	Nonsignificant

Then, we are conducting a Kruskal–Wallis test for COL10 data to determine whether there is a significant difference between treatments and a considerable difference between one treatment and another. Based on the analysis results, a p < 0.0001 means a significant difference between the dose at the 95% confidence level (Table 3).

The comparison between each group after the Kruskal–Wallis test was carried out, followed by using the Mann–Whitney test. The results of the Mann–Whitney test that the dose has a difference between groups is shown if it has a p < 0.05 (Table 5).

Table 5: Mann–Whitney test results

Difference dosage	Average difference	p	Information
0			
0.1 mg/ml	19.303	0.009	Significant
1 mg/ml	17.477	0.009	Significant
2 mg/ml	−0.206	0.009	Significant
3 mg/ml	18.142	0.009	Significant
0.1 mg/ml			
1 mg/ml	−1.826	0.009	Significant
2 mg/ml	−19.509	0.009	Significant
3 mg/ml	−1.161	0.009	Significant
1 mg/ml			
2 mg/ml	−17.682	0.047	Significant
3 mg/ml	0.665	0.028	Significant
2 mg/ml			
3 mg/ml	18.347	0.009	Significant

The results of the Mann–Whitney test, COL10 comparison between other doses have a significant difference in collagen levels. Furthermore, the researchers conducted a correlation test between the dose with COL2 and COL10, this purpose of determining whether there is a relationship or not that can be seen from the significance or (p) value and how strong the relationship can be seen from the correlation coefficient value or (r).

The relationship between dosage – COL2 is known that the correlation value is 0.665 with a p of 0.000, the correlation value of 0.665 indicates that the dose with COL2 has a strong relationship, and with a positive relationship suggests that the higher the dose will give an increase in level COL2. Meanwhile, with p = 0.000 < 0.05 (α = 5%), it can be concluded that there is a significant relationship between the dose HA and COL2. However, the relationship between dosage – COL10 is known that the correlation value is -0.335 with a p of 0.101, the correlation value of 0.335 indicates that the dose with COL10 has a weak relationship and with a negative relationship suggests that the higher the dose will decrease COL10 level. Meanwhile, with p = 0.101 > 0.05 (α = 5%), it can be concluded that there is a nonsignificant relationship between the dose HA and COL10 (Table 3).

Discussion

In OA conditions, COL2 will undergo two main mechanisms: anabolic and catabolic, with some of the chondrocytes in cartilage will undergo terminal differentiation into hypertrophic-like chondrocytes. Hypertrophic-like chondrocytes will express extracellular matrix in the form of COL10, which is a specific marker. Meanwhile, normal chondrocytes that do not undergo differentiation will still express COL2 even with a decreased concentration compared to normal cartilage conditions. This condition is caused by the unbalanced anabolic and catabolic responses of COL2, especially in states of severe cartilage damage [13]. Meanwhile, a high level of COL2 as an extracellular matrix, especially in cartilage, helps improve structural integrity in cartilage and helps the body respond to joint cartilage repair.

In this study, there was a decrease in COL2 level in the control group, and after receiving a 0.1 mg/ml dose of HA in the treatment group, there was still no increase in the expression of COL2. However, with a dose of 1 mg/ml, a significant increase in COL2 was seen. The different finding is shown with the higher dose of HA, with 2 mg/mL and 3 ml/mL, there was no further increase of COL2. This result is in parallel with the research of Alleman *et al*., in an in vitro experiment where an HA scaffold was inserted into the chondrocyte culture environment; the results of measurements using polymerase chain reaction showed that administration of HA scaffold with a dose of 2% could increase the mRNA

Strobe Statement — Checklist of items that should be included in reports of cross-sectional studies

Item Details	Item No	Recommendation	Page No	Relevant text from manuscript
Title and abstract	1	*(a)* Indicate the study's design with a commonly used term in the title or the abstract	2	This research is *in vitro* experimental research (pre-and posttest control group design)
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	2	In the point of the objective and the result
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3−4	
Objectives	3	State-specific objectives, including any prespecified hypotheses	4	We hypothesized
Methods				
Study design	4	Present key elements of study design early in the article	5	Sample collection and data acquisition
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5−6	
Participants	6	*(a)* Give the eligibility criteria and the sources and methods of selection of participants	5	The inclusion criteria were….
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5	Sample collection
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	7	
Bias	9	Describe any efforts to address potential sources of bias	6	To prevent bias in this study….
Study size	10	Explain how the study size was arrived at	5	20 Sample
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why		
Statistical methods	12	*(a)* Describe all statistical methods, including those used to control for confounding	7	Statistical analysis
		(b) Describe any methods used to examine subgroups and interactions	7	Statistical analysis
		(c) Explain how missing data were addressed		No missing data
		(d) If applicable, describe analytical methods taking account of sampling strategy	5	Sample
		(e) Describe any sensitivity analyses	7	Statistical analysis
Results				
Participants	13*	(a) Report numbers of individuals at each stage of the study — e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analyzed	7−9	Result
		(b) Give reasons for nonparticipation at each stage		No samples are excluded
		(c) Consider the use of a flow diagram		
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, and social) and information on exposures and potential confounders	8	
		(b) Indicate the number of participants with missing data for each variable of interest		
Outcome data	15*	Report numbers of outcome events or summary measures	17−19	Table
Main results	16	*(a)* Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	8−9	Results
		(b) Report category boundaries when continuous variables were categorized		
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period		
Other analyses	17	Report other analyses done — e.g., analyses of subgroups and interactions and sensitivity analyses		
Discussion				
Key results	18	Summarize key results with reference to study objectives	10−12	Discussion
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	11	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	10−11	
Generalizability	21	Discuss the generalisability (external validity) of the study results	11	
Other information				
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	14	Role of the funding source

*Give information separately for exposed and unexposed groups. An explanation and elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

expression of COL2 up to four times. However, the same study also showed that giving HA levels that are too large, at a dose of 10%, would inhibit the expression of COL2 [14]. Therefore, data concluded that the dose of 1 mg/mL is the optimal dose that could increase the expression of COL2. In addition, it is necessary to pay attention to the optimal and not excessive dose of HA so that it does not inhibit the expression of COL2.

HA also inhibits the effect of IL-1β and NO on the activation of the RUNX2 gene, thereby inhibiting the differentiation process of terminal chondrocytes (hypertrophic-like chondrocytes) [15]. Furthermore, this inhibition will make the number of hypertrophic-like chondrocyte cells do not increase but decrease due to the tendency of these cells to experience apoptosis.

A specific marker for hypertrophic-like chondrocytes is COL10. Normal chondrocytes cannot extract this specific substance in human joint cartilage. Therefore, COL10 levels would be detected at protein and mRNA levels in human cartilage with OA. COL10 levels will be found higher in OA, which suffered from more severe tissue degradation [16]. There is an association between COL10 levels and the severity of OA. The higher the level of COL10 indicates, the more profound the gap in the cartilage and the more exposed the subchondral bone [17].

Oxidative stress and inflammatory reactions are also occurring in the OA culture sample, therefore increasing terminal differentiation and hypertrophic-like activity of chondrocytes as evidenced by increased levels of COL10. The increased hypertrophic-like activity of chondrocytes will increase levels of MMPs, especially MMP-13, an enzyme capable of breaking down extracellular matrices and aggravating cartilage damage in individuals with OA [17].

In this study, a decrease of COL10 expression was significant among all doses of HA. In addition, the negative relationship between the dose and COL10 in

Spearman's correlation test showed that administering a more significant amount of HA would reducing COL10. In other words, every additional milligram dose of HA will suppress the expression of COL10. This theory follows the theory that HA plays a significant role in suppressing COL10 and provides a protective effect against OA [18], [19].

For future research, the authors suggest that in vivo studies with more variables such asACAN, SOX9, and other substances can be carried out to observe HA administration's biological responses better.

Conclusion

The administration of HA can increase COL2 and decrease COL10, so it has the potential function of inhibiting the degeneration process in joint cartilage. The optimal dose of HA found in this study was 1 mg/mL.

The Point in this Study Adds

1. The optimal effective dose of HA for the chondroprotective effect based on COL2 and COLX levels is 1mg/mL.
2. The higher dose of HA did not increase COL2 further than 1mg/mL.
3. Administering a higher dose of HA significantly suppresses the COL10 even more.

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