Chondrotoxicity induced by intra-articular injection of corticosteroid in human articular cartilage

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Doctoral Dissertation submitted to
the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

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December 2010

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December 2010

ACKNOWLEDGEMENTS

Doctoral study is a harvest of positive influences from many supervisors that lend their personal efforts and expertise. Firstly, I would like to express my gratitude to my supervisor, Professor Sung-Jae Kim, for his encouragement and support throughout this research. His enthusiastic and rigorous approach to medical science helped me appreciate what it takes to be a good doctor and an exceptional researcher.

I am deeply indebted to Professor Jin Woo Lee and researcher Ho-Sun Jung for their extraordinary support throughout my investigations. I would also like to extend my appreciation to my committee members, Professor Nam-Hong Choi, Professor Jong-Chul Park and Professor Young-Bum Park for their valuable detailed comments and suggestions.

Further, I owe my deepest gratitude to my friends, Eric Shih and Jimmy Chang for their expertise and language consulting. I am blessed that I have the support of my wonderful family: my parents, my wife, Hai-Sun Lee and children, Michelle and Justin.

Finally, my deepest gratitude goes to God who governs the world among the individuals.

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ABSTRACT

Chondrotoxicity induced by intra-articular injection of corticosteroids in human articular cartilage

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INTRODUCTION: When a corticosteroid is injected into osteoarthritic knees to decrease synovial swelling, joint effusion and pain, the articular cartilage of the injection site is also exposed to the drug simultaneously. The adverse effect of corticosteroids on chondrocyte of the articular cartilage is a concern. Thus, the number of injections is normally restricted to no more than four times a year. This work is mainly focused on the evaluation and comparison of chondrotoxicity of two commonly used corticosteroids: triamcinolone acetate (TA) and dexamethasone disodium phosphate (DE).

MATERIALS AND METHODS: Fresh articular chondrocytes from human knees (degenerative osteoarthritis patients) were collected and incubated with various concentrations of TA or DE for 3 days. The cell proliferation rate was investigated with methylthiazoletetrazolium (MTT) assay and the results were compared with that of the control group (culture media and 0.9% saline solution). Distribution of apoptotic and necrotic cells in samples as well as cell cycle were analyzed using fluorescence-activated cell sorting (FACS) . Glycosaminoglycan (GAG) secretion in the study samples was measured photometrically using sulphated GAG assay. All measurements were

performed two days after each sample was exposed to the study drugs with and without 10ng/ml of IL-1β. All samples were tested in triplicates and statistical significance was determined using Student's t-tests with $p<0.05$ considered significant.

RESULTS: The proliferation of chondrocytes was inhibited during three days of exposure in the TA groups with concentrations higher than 31.25 μg/ml (groups TA1 to TA5) ($p<0.02$). All the DE groups with drug concentrations lower than 500 μg/ml failed to inhibit the proliferation of chondrocytes from day 1 to day 3, whereas 500 μg /ml of DE inhibited the chondrocyte proliferation. Among them, the group with the highest concentration of DE below 500 μg/ml was 62.5 μg/ml. A significant decrease in GAG secretion was observed in TA4 (62.5 μg/ml) group ($p=0.003$) while TA7 (3.125 μg/ml) and DE groups did not affect GAG secretion. Apoptosis was not detected in group TA4 which inhibited proliferation of chondrocyte as well as group TA7 which showed normal proliferation of chondrocyte. The proliferation of 10ng/ml IL-1β treated chondrocyte was inhibited significantly in all TA groups ($p<0.02$) compared to the control group. The GAG secretion of IL-1β treated chondrocyte decreased significantly ($p<0.05$) in all TA and DE groups compared to the control group. While the contents were increased upon dilution of TA concentration, the peak content in group TA5 decreased again.

CONCLUSION: Dexamethasone disodium phosphate could be considered as a safer corticosteroid for frequent repeated intra-articular injection than triamcinolone acetate with regard to chondrotoxicity. A more frequent intra-articular injection of low concentrations of dexamethasone disodium phosphate might be a viable new treatment strategy for osteoarthritic knees.

Key words: chondrotoxicity, triamcinolone acetate, dexamethasone disodium phosphate, intra-articular injection, knee

Chondrotoxicity Induced by Intra-articular Injection of Corticosteroid in Human Articular Cartilage

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I. INTRODUCTION

Intra-articular (IA) injection of corticosteroids is an effective treatment method for relieving pain, swelling and joint effusion of arthritic knees. It is a frequently used and widely accepted as a preferred treatment by both physicians and patients when typical medications and physical therapies do not relieve the symptoms. Its results are superior to that of systemic use of corticosteroid[1].

Many studies suggest that the treatment effect of injected corticosteroid is acting on the affected synovium rather than the articular cartilage of a knee joint. There are studies that also suggest when internal structures of knee joint is exposed to frequent intra-articular injection of high doses of corticosteroids, it has a detrimental effect on anterior cruciate ligament[2], meniscus[3] and articular cartilage[4-7]. Despite the fact that corticosteroids could potentially induce undesired side effects on articular cartilage, this procedure is still preferred by most physicians when the benefits of intra-articular injection of corticosteroids outweigh the risks of chondrotoxicity for patients.

Intra-articular injection of corticosteroid can be traced back as early as 1950s. Its potential application for rheumatoid arthritis and osteoarthritis were first demonstration by Hollander in 1951[8], and Miller et al in 1958[9], respectively. Less than a decade later, the adverse effects of this treatment were reported and referred to as "steroid arthropathy"[6,7]. Most of corticosteroid studied at the time were weekly injection of cortisone and hydrocortisone[6,10].

Since 1960, uses of more potent, longer acting synthetic steroids for intra-articular injection were studied. Firstly, methylpredinisolone acetate was found to have a better efficacy and safety profile than other corticosteroids when it was used at lower number of injections due to its higher relative glucocorticoid potency (RGP) and longer duration of action. Murdoch[11] found that methylprednisolone could last in the knee joint cavity for as long as 21 days while hydrocortisone is completely cleared in the joint cavity within 24 hours. Triamcinolone is another commonly used intermediate-acting corticosteroid. The RGP of triamcinolone compared to cortisol is five, which is same as methylprednisolone, and the duration of action is longer than that of methylprednisolone[12]. Results of studies on another triamcinolone analog, triamcinolone hexacetonide (THA) have been reported since 1967[13]. THA is believed to be more effective than TA[14,15] in intra-articular injection, but it is not commercialized in some countries including Korea. While there are corticosteroids (dexamethasone and betamethasone) with characteristics of higher RGP and longer duration of action than triamcinolone and methylprednisolone[12], their use as intra-articular injection has not been extensively studied.

According extant literatures on osteoarthritis, THA is clinically more effective and has longer residence time in knee joint than bethamethasone[16]. THA is also more effective in early pain reduction than methylprednisolone acetate (MPA), even though MPA has longer effect lasting up to eight weeks[17]. TA has similar effect of MPA at one fourth of dose[18].

Besides the efficacy of corticosteroids, there is a regional preference of

corticosteroids. Centeno[19] conducted a survey of the members of American College of Rheumatology (ACR) on their preference and choice of intra-articular corticosteroid injections. The survey concluded that the corticosteroids favored by respondents depended on place of their training. TA was preferred by those trained in the western United States, THA was preferred by those trained in the Midwest and Southwest, while MPA was preferred by those trained in East.

While there are numerous published studies comparing clinical effectiveness of corticosteroids and practitioners' preference for use of one corticosteroid over another, no study has ever been conducted comparing safety of intra-articular corticosteroid injections. The main focus of this research is to evaluate and compare chondrotoxicity of triamcinolone acetate (medium-acting) suspension and dexamethasone disodium phosphate (long-acting) solution.

II. MATERIALS AND METHODS

1. Articular chondrocyte isolation and cell culture

After approval from the Institutional Review Board (IRB), fresh articular cartilage surgical waste specimen harvested during total knee replacement (TKR) of degenerative osteoarthritis patients (aged 55-73 years) were used to prepare articular chondrocyte used in this study. These specimens were collected from 10 patients where 7 of 10 patients had simultaneous bilateral sequential TKR and remaining 3 patients had unilateral TKR. Patients with rheumatoid arthritis were excluded. We collected the full thickness normal appearing cartilage slices from the articular surface of distal femur and proximal tibia with sterile No. 10 blade. To disjoin chondrocytes from the ECM, the cartilage was incubated at 37°C, 5% CO_2 in enzymatic digestion media containing collagenase II (10 mg/1 g of cartilage) and hyaluronidase (6 mg/1 g of cartilage, Worthington Biochemical, Lakewood, NJ) in Dulbecco's modified Eagle's medium (DMEM) - high glucose (Gibco BRL, Grand Island, NY) with 1% antibiotic-antimycotic (Gibco BRL). The isolated cells were strained by a 100 μm nylon cell strainer (BD Falcon, Bedford, MA), pelleted by centrifugation and resuspended in growth media (DMEM-high glucose containing 10% fetal bovine serum (FBS, Gibco BRL) and 1% antibiotic-antimycotic).

After 1 week in culture, chondrocytes were segregated into groups: chondrocytes exposed to triamcinolone acetate (TA) or dexamethasone disodium phosphate (DE), and chondrocytes activated with 10ng/ml of IL-1β and exposed to triamcinolone acetate (TA) or dexamethasone disodium phosphate (DE).

2. Cell proliferation assay

The cell proliferation rates were investigated by the methylthiazoletetrazolium (MTT) assay. Cells were seeded in 48-well plates at a density of $1x10^4$ cells per well and incubated with TA or DE for 3 days. Concentrations of TA or DE are described in Table 1.

Table 1. Concentration of Triamcinolone acetate and Dexamethasone disodium phosphate

Group	Triamcinolone acetate (TA) (ug/ml)	Dexamethasone disodium phosphate (DE) (ug/ml)
1	500	62.5
2	250	31.25
3	125	15.625
4	62.5	7.8125
5	31.25	3.90625
6	15.625	1.953125
7	3.125	0.390625

The culture media was removed and 300 μL of fresh media was added to each well of 48-well plate. Fresh MTT solution (Sigma, St. Louis, MO, USA) 200 μL of 0.5 mg/mL was added to the culture plates and incubated for 4 hours. The media was then removed again, and intracellular formazan was solubilized by adding 400 μL of dimethylsulfoxide (DMSO, Sigma) into each well. The absorbance of the formazan product was measured in triplicate at 570 nm by spectrophotometric microplate reader.

The supplementary chondrocyte proliferation assay was performed with 8, 4, 0.5 mg/ml of TA suspensions and 1, 0.5, 0.0625 mg/ml of DE solutions. For TA samples, the assay was performed after filtering out suspension particles.

3. Analysis of apoptosis by flow cytometry

The ApoScan™ Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection Kit (BioBud, Korea) was used to detect apoptosis by flow cytometry. Chondrocyte were plated onto 100 mm dishes and incubated with TA or DE for 2 days. Cells were trypsinized, harvested, and resuspended in DMEM. Cells were washed in cold phosphate buffered saline (PBS) and pelleted by centrifugation at 1000 rpm for 5 minutes. They were then resuspended in a 1×binding buffer in DMEM. HepG2 cells were washed in cold phosphate buffered saline and pelleted by centrifugation at 1,000 ×g for 5 min. They were then resuspended in a 1×binding buffer (500 μL) and incubated with 1.25 μL of Annexin V-FITC (200 μg/mL) at room temperature for 15 minutes. The cells were resuspended in 500 μL of a 1×binding buffer and then cell suspensions were stained with 10 μL of PI (30 μg/mL) at 4℃ in the dark. The fluorescence was determined using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA). A computer system of CellQuest Program (Becton Dickinson) was used for data acquisition and analysis.

4. Cell cycle

Cells were harvested by incubation with 0.25% trypsin/EDTA and washed with PBS two times. 106 cells from each group were fixed with dry ice-cold 70% ethanol for 1 hour at -20℃, stained with 50 μg/mL of propidium iodide (PI, Sigma) containing 100 μg/mL of RNase A (Sigma) for 40 minutes at 4℃, and then measured by FACS Calibur (Becton Dickinson) to detect cell cycle distribution. All samples were tested in triplicates (n=3).

5. Sulfated glycosaminoglycan (GAG) assay

The secreted GAG content was determined using the Blyscan Kit (Biocolor Ltd, Newtown Abbey, Northern Ireland, UK) according to the manufacturer's instructions. Briefly, an equal volume of media was mixed with the Blyscan Dye Reagent by shaking for 30 min to saturate the GAG-dye binding. After centrifugation, the dye bound to GAGs was dissolved in Dissociation Reagent, and the recovered dye concentration was determined photometrically by measuring the absorbance at 656 nm. A chondroitin-4-sulfate standard solution (Biocolor) was used to generate the standard curves, and all samples were tested in triplicate.

6. RT-PCR

The total RNA was isolated from primary chondrocytes using the RNeasy kit (Qiagen, Valencia, CA, USA). The total RNA was reverse-transcribed using the Omniscript kit (Qiagen) and all experiments were performed with Taq DNA polymerase (Qiagen).

The reaction products were subjected to 1.5% agarose gel electrophoresis. Primer sequences specific for amplication of RT-PCR was used for amplification of genes encoding GAPDH, type I collagen, type II collagen, type X collagen were listed in Table 2.

Table 2. Primer used for RT-PCR assay of primary chondrocyte

Target gene	Primer
GAPDH 1F	GAAGGTGAAGGTCGGAGTC
GAPDH 1R	GAAGATGGTGATGGGATTTC
TYPE I Collagen 1F	AGACATCCCACCAGTCACCT
TYPE I Collagen 1R	GTGGGTGACACCTCGCCTTC
TYPE II Collagen 1F	TTCAGCTATCCAGATGACAATC
TYPE II Collagen 1R	AGAGTCCTAGAGTGACTGAG
TYPE X Collagen 1F	GCCCAAGAGGTGCCCCTGGA
TYPE X Collagen 1R	CCTGAGAAAGAGGAGTGGAC

7. Cell proliferation rate, cell cycle and sulphated GAG assay with IL-1β treatment

Same measurements were performed two days after each sample was exposed to the study drugs with 10ng/ml of IL-1β.

8. Statistical analysis

All results are presented as the average ± standard deviation. Statistical significance was determined using Student's t-tests with values of $p<0.05$ considered as statistically significant.

III. RESULTS

We determined typical phenotype of primary chondrocyte after one week of culture. RT-PCR assay showed marked expression of type II collagen which mainly comes from articular cartilage. There was no expression of type I collagen which is usually found in bone tissue and type X collagen. Type I collagen is recognized as hypertrophic marker (Fig. 1).

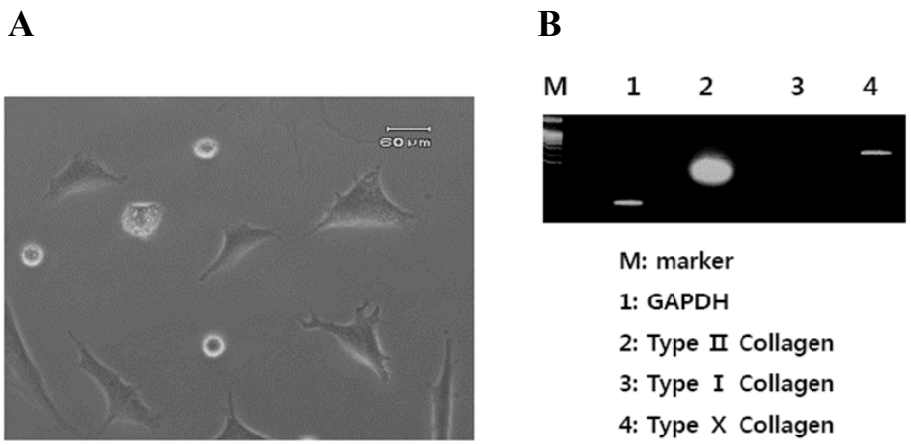

Fig. 1. Chondrocytes phenotypes and specific marker gene expression. (A) Primary chondrocytes were round shape. (B) The expression of Type I collagen, Type II collagen, and Type X collagen was analyzed after one week of culture. Analysis of Type I collagen, Type II collagen, and Type X collagen expression levels were investigated by RT-PCR. GAPDH was used as an internal control.

1. Effects of TA and DE on chondrocyte viability

During the three days of exposure, the cell proliferation of chondrocytes was not noticed in any TA groups with concentrations higher than 31.25 μg/ml (groups TA1 to TA5). There were significant difference in cell count between each of these groups and control group ($p<0.02$). In groups TA6 and TA7, the proliferation of chondrocytes increased from day 1 to day 3 and showed the same distribution as control group (media and 0.9% normal saline group). All the dexamethasone groups (groups DE1-DE7) fail to inhibit the proliferation of chondrocytes and showed similar pattern of proliferation from day 1 to day 3 as that of control group (Fig. 2).

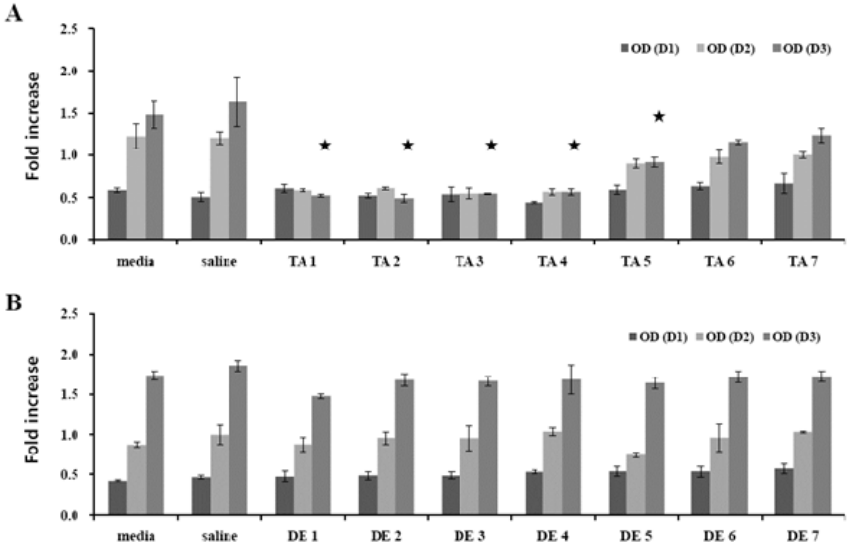

Fig. 2. Effects of chondrocytes proliferation with 1 day, 2 days, 3 days of continuous exposure to TA and DE. (A) Triamcinolone acetate treated groups. (B) Dexamethasone disodium phosphate treated groups. ★ : $P < 0.02$ compared with media and normal saline solution.

In high concentrations of filtered TA solution groups, the cell proliferation of chondrocytes was inhibited in TA 8 mg/ml and TA 4 mg/ml groups, but not in TA 0.5 mg/ml group. Likewise, the proliferation of chondrocytes was inhibited in DE 1 mg/ml and 0.5 mg/ml groups, but not in 0.0 625 mg/ml group (Fig. 3).

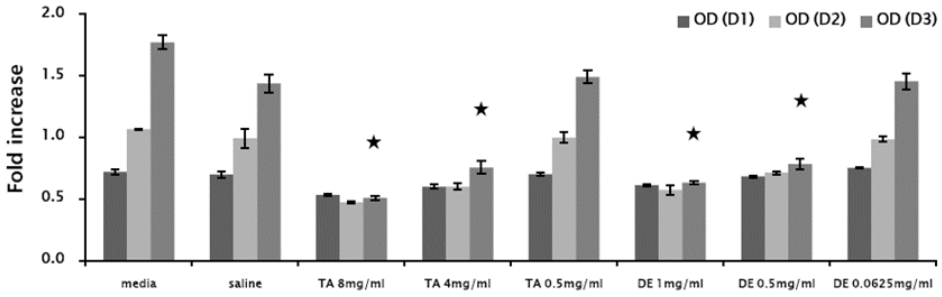

Fig. 3. Effects of chondrocytes proliferation with 1 day, 2 days, 3 days of continuous exposure to high concentration of filtered TA suspension and DE solution. ★ : $P < 0.01$ compared with media and normal saline solution.

2. Analysis of apoptosis and necrosis

Among DE groups, no apotosis or necrosis were observed in DE4 and DE7 groups. In TA groups, there were no apoptosis or necrosis in TA4 which inhibited proliferation of chondrocyte as well as TA7 which showed normal proliferation of chondrocyte (Fig. 4).

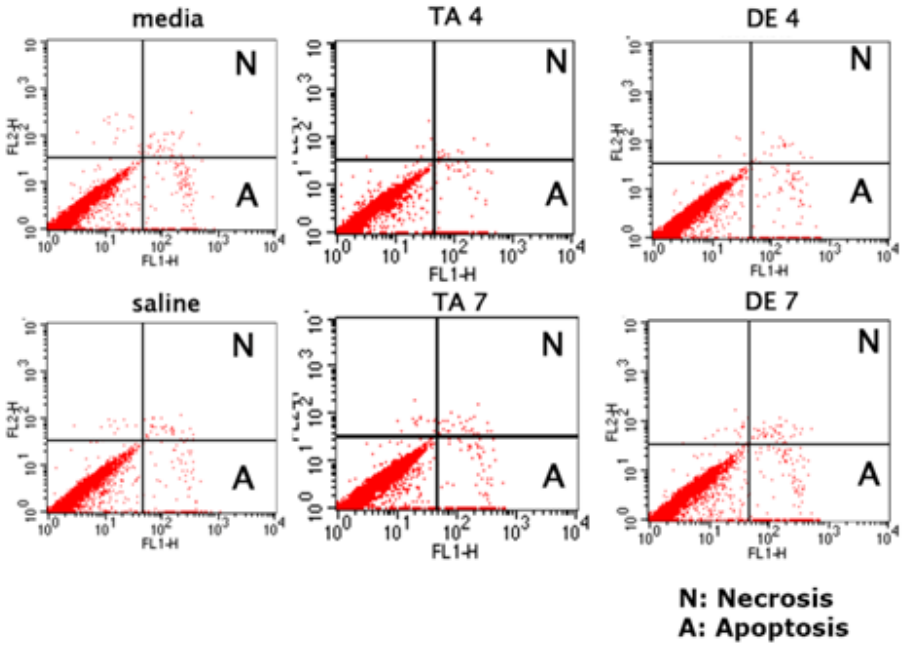

Fig. 4. Scatter plots 2 days after media, 0.9% normal saline solution, TA4 (62.5 μg/ml), TA7 (3.125 μg/ml), DE4 (7.81 μg/ml) and DE7 (0.39 μg/ml). Propidium iodide fluorescence (ordinate) is plotted against annexin V fluorescence (abscissa).

3. Analysis of cell cycle

All the glucocorticoid treated groups exhibited a proportional increase of G2/M phase cells in their cell cycles when compared to the control group. There was no significant difference between each group and control group. Distribution of G2/M phase cells in media and saline group was the same (Fig. 5).

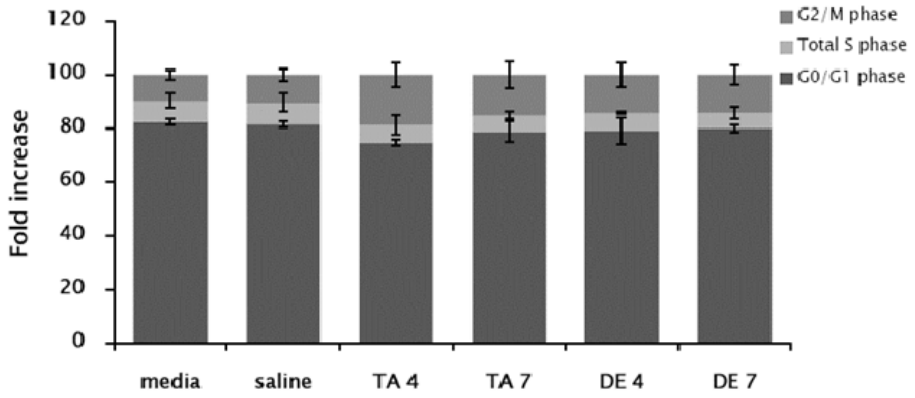

Fig. 5. Chondrocyte cell cycle 2 days after exposure to media, 0.9% normal saline solution, TA4 (62.5 µg/ml), TA7 (3.125 µg/ml), DE4 (7.81 µg/ml) and DE7 (0.39 µg/ml). Treated cells harvested and then 10^6 cells. Each experiment was carried out in triplicates.

4. Analysis of GAG content

A significant decrease in GAG secretion was observed in TA4 group (p=0.003). The GAG content of groups TA7, DE4 and DE7 were comparable to that of control group (Fig.6).

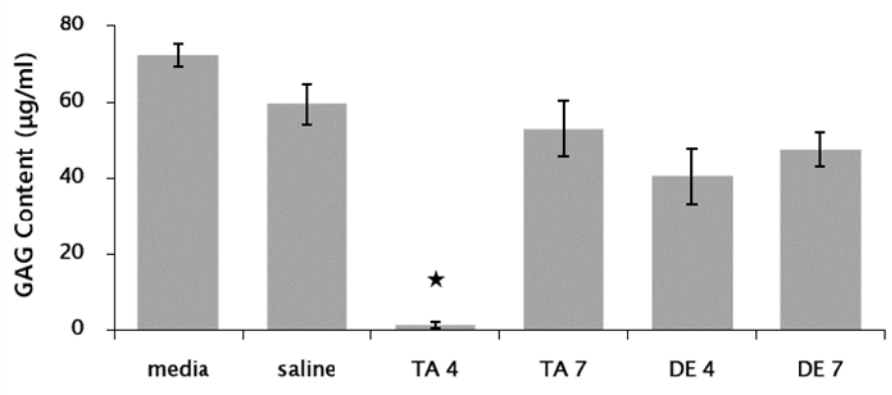

Fig. 6. Effect of GAG secretion in chondrocyte. Cells were treated in media, 0.9% normal saline solution, TA4 (62.5 μg/ml), TA7 (3.125 μg/ml), DE4 (7.81 μg/ml) and DE7 (0.39 μg/ml) for 2 days. Concentration of dissolved GAGs was determined photometrically at 656 nm using standard curves of chondroitin-4-sulfate standard solution (Biocolor). Results are represented as the mean ± SD of triplicate wells from three independent experiments. ★ : $P < 0.01$ compared with media and normal saline solution.

To verify the induced inflammation by IL-1β on our chondrocytes, Western blot analysis was applied. The cyclooxygenase-2 (Cox-2) expression was detected by recombinant human IL-1β in human chondrocytes (Fig. 7).

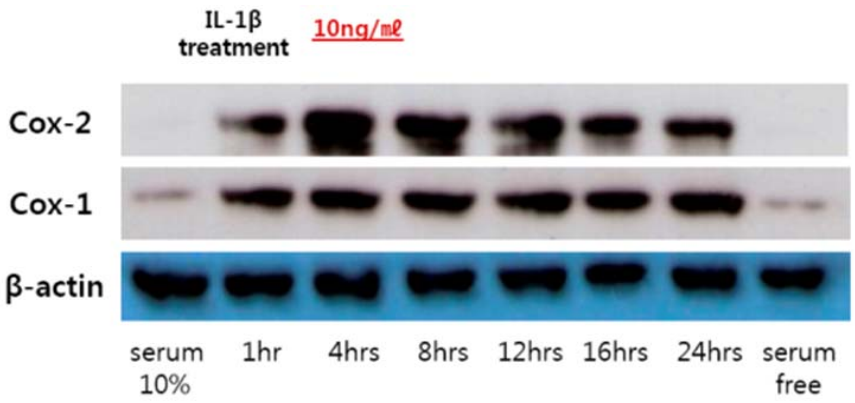

Fig. 7. Induction of cyclooxygenase-2 by recombinant human IL-1β in human chondrocytes. Western blot analysis was performed to determine the most suitable concentration of IL-1β for 24 h in chondrocytes. The COX-2 expression was not affected by 10 ng/mL of IL-1β. It was enhanced at 4 to 8 h and slowly decreased over 12 h by 10 ng/mL of IL-1β. However, the COX-1 was expressed constantly regardless of IL-1β concentration. The above data has been confirmed on samples from all 3 donors.

5. Effects of TA and DE on IL-1β treated chondrocyte viability

Significant inhibition of chondrocyte proliferation was noticed in all triamcinolone groups ($p<0.02$) and all dexamethasone groups ($p<0.05$), but there was an increasing number of viable chondrocytes from day 1 to day 3. Particularly in TA6 and TA7, the daily increase in cell was remarkable, as well as all the dexamethasone groups (Fig. 8).

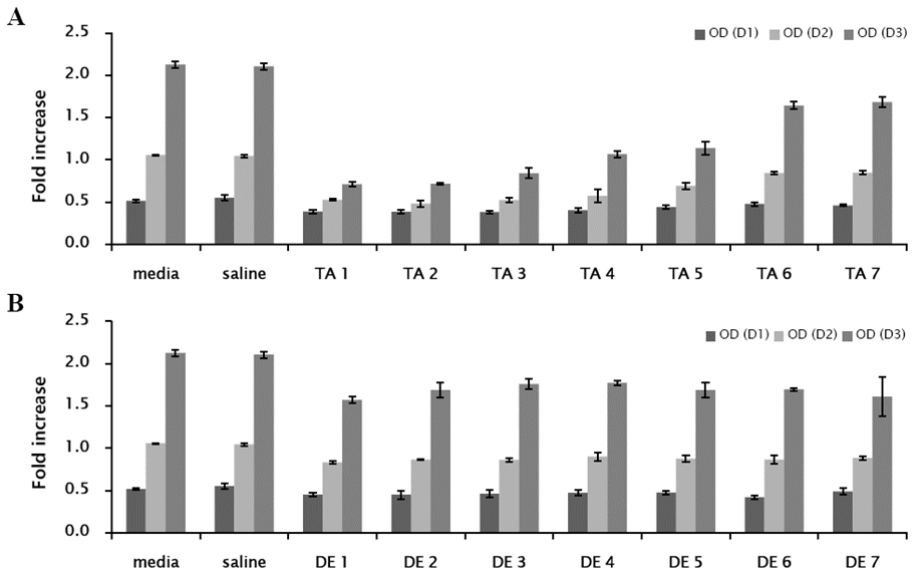

Fig. 8. Effects of IL-1β (10 ng/ml) on chondrocytes proliferation with 1 day, 2 days, 3 days of continuous exposure to TA and DE. (A) Triamcinolone acetate exposed groups. $P < 0.02$ compared with media and normal saline solution in all TA groups. (B) Dexamethasone disodium phosphate exposed groups. $P < 0.05$ compared with media and normal saline solution in all DE groups.

6. Analysis of cell cycle of IL-1β treated chondrocyte

Proportional decreasing of S phase cells was measured in TA4, TA7, DE4 and DE groups, but it was not statistically significance (Fig. 9).

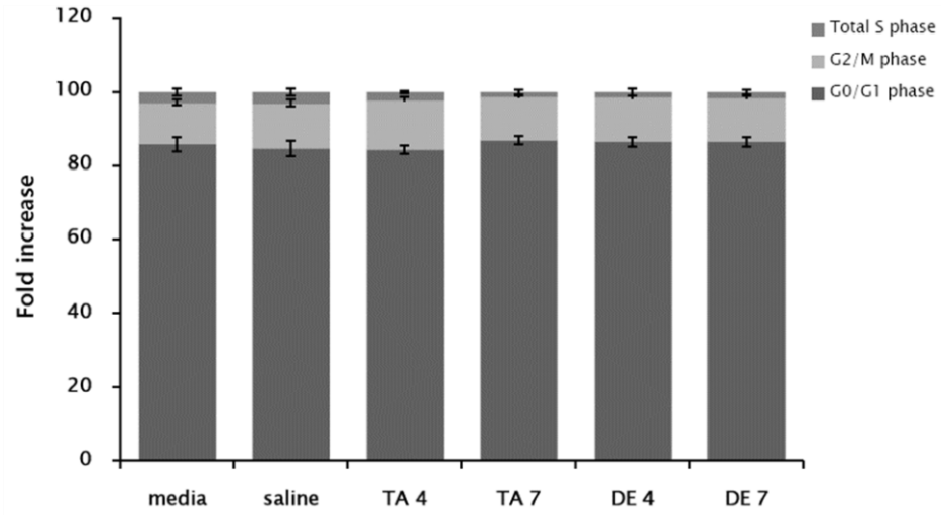

Fig. 9. Cell cycle distribution of IL-1β treated chondrocyte measured by FACS Calibur. Chondrocyte cell cycle 2 days after exposure to media, 0.9% normal saline solution, TA4, TA7, DE4 and DE7 with 10ng/ml IL-1β. Each experiment was carried out in triplicate.

7. Analysis of GAG content of IL-1β treated chondrocyte

GAG secretion decreased significantly in all TA groups and DE groups ($p<0.05$). Upon the dilution of TA concentration, the GAG content initially increased from TA1 to TA5. After reaching its peak at TA5, it declined in TA6 and again in TA7 (Fig. 10).

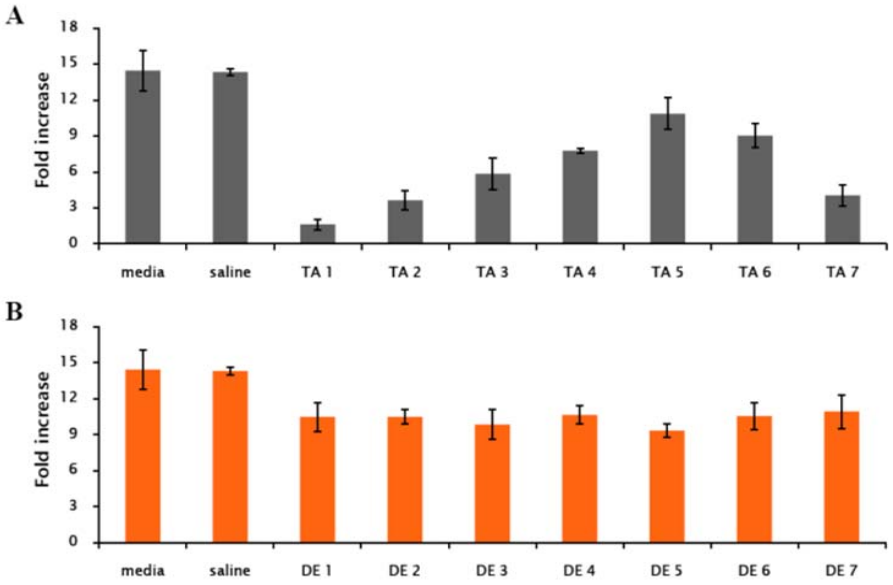

Fig. 10. Effect of GAG secretion in 10ng/ml of IL-1β treated chondrocyte. Concentration of dissolved GAGs was determined photometrically at 656 nm using standard curves of chondroitin-4-sulfate standard solution (Biocolor). $P < 0.05$ compared with media and normal saline solution in all TA and DE groups.

IV. DISCUSSION

The present study focused on the evaluation of chondrotoxicity of triamcinolone (TA) and dexamethasone (DE) when injected into the knee joints for degenerative osteoarthritis treatment. Osteoarthritis is a relatively non-inflammatory condition compared to rheumatoid arthritis, but the degree of non-erosive synovitis of osteroarthritic knees is believed to be secondary to the cartilage breakdown which inflames synovium to increase synthesis of proteases and they may break down cartilage matrix[20,21]. An intra-articular glucocorticoid injection is perceived to be effective in treating osteoarthritis and has lesser systemic toxicities of corticosteroids.

Regarding to systemic toxicity of corticosteroids, many investigations demonstrated intra-articular steroid injection, suppresses hormone production of the hypothalamus-pituitary-adrenal (HPA) axis. While the influence is short term and reversible, but it is significant. The plasma cortisol level usually returns to the normal level within 1-2 weeks after IA injection of MPA[22] and betamethasone[23], and 2-4 weeks after injection of TA[24]. The iatrogenic Cushing syndrome could be resulting from repetitive injection[25]. IA steroid injection decrease marker of bone turnover and bone formation but it does not affect bone resorption marker[26]. Transient facial flushing and hyperglycemia are other reported systemic side effects which can last for 1-2 days [27,28].

Among local toxicities, cartilage damages and infections resulting from repeat IA steroid injections are most problematic. The steroid induced self-limiting synovitis (steroid flare) can happen and lasts for up to 48 hours[29]. Fuenfer et al[30]. have observed that steroids depress bactericidal activity of neutrophils. Likewise, Armstrong et al[31] reported that the likelihood of septic arthritis after arthroscopy surgery is 20 times greater when corticosteroids were used. They also commented, in a separate series, that intra-articular injection of methylprednisolone acetate is the

most significant risk factor for post arthroscopic infection [32].

Balch HW et al[33]. recommended in 1977 that the interval between steroid injections should not be less than one month. According to American College of Rheumatology (ACR) subcommittee on rheumatoid arthritis guidelines issued in 1995, the injection of steroids in a same joint should not be done more than once within 3 month period. This position had not change in the 2002 update[34].

Due to known concerns of frequent steroid injection, most clinicians rely on conservative dosing guideline (three month interval) provided by the ACR. A randomized double-blinded study showed the safety of long-term (every 3 months for up to 2 years) intra-articular TA (1 vial, 40mg) injection compared with placebo group[35]. But based upon pooled data from the meta-analyses[36,37], the significant effect of corticosteroid IA injection on pain relieve is up to 4 weeks after injection, and there is no difference between IA injection and control groups by 6 to 8 weeks after injection. The dosing frequency provided in the ACR guideline has been questioned to be too conservative and clinicians have been beseeching a safe guideline on shorter interval of injection for intractable patients. Roberts WN et al[38]. reported that the frequent injection (more than 4 injections per year in a single joint) does not increase the rate of total knee replacement in rheumatoid arthritis.

Inquiring further into the influence of corticosteroids on articular cartilage, many experiments have demonstrated matrix loss and decreased proteoglycan synthesis[4, 5], whereas other studies have reported that glucocorticoids are chondroprotective under specific conditions[39-41]. In present experiment, the significantly decreased GAG content was detected in group TA4 but not in group TA7. However, these findings might represent inhibitory effect of TA in proteoglycan synthesis, which is known to be dose-dependent.

Moreover, most candidates of osteoarthritic knees for steroid IA injection are aged population. Their cartilage showed different pattern of protein secretion and metalloproteinase activity from that of younger cartilage[42]. The age related

difference in the response to corticosteroid was noted[43] and Livne et al[44] found that DE stimulated rather than depressed the cellular proliferation in arthritic cartilage. In our investigation, the proliferation of IL-1β treated chondrocyte was inhibited in higher concentration of group TA5, but the increasing number of viable chondrocyte was noticed from day1 to day 3 while those of chondrocytes without IL-1β stimulation almost stayed same. Further study will be needed to find whether the susceptibility of chondrotoxic effect by corticosteroid is lower in inflamed cartilage. If it is it, the concern of cartilage damage of corticosteroid injection could be lessened for osteoarthritic knees compared normal knees where injection is not usually prescribed.

To minimize the frequency of injection, we should find a way to improve the efficacy of each single injection. Proper needle placement is an important aspect. Aspiration of synovial fluid is also generally recommended at the time of injection. Resting of affected joint for 24 hours after injection was proposed by some studies[19,45]. Cold packs may be used also for delaying absorption of the corticosteroid in the injected knee joint. Above and beyond these factors, the kind of agents and formulations of glucocorticoid which last longer in the affected joint cavity might able to reduce the frequency of injection. The residence time of an intra-articular injected agent in the knee joint mainly depends on its solubility and dissolution rate. Poorly soluble agents applied as suspension formulations last longer in the knee joint than the soluble glucocorticoids applied as solution formulations[46,47]. Thus, DE may have shorter residence time than TA, even the half life of DE is longer than that of TA. The effective acting time of DE on synovium of the knee may be shorter than TA. Once DE is cleared from the knee joint and absorbed into the blood stream through synovium, the influence on HPA axis and systemic effect may or may not last longer than TA. If the residence time of TA in knee joint cavity was longer than DE, it means that the exposure time of TA to articular cartilage is longer. This might increase concerns of potential chronotoxicity.

However, because the arthropathy is a result of repetitive cumulative effect, drug with shorter residence time which is absorbed faster could be the less chondrotoxic drug for steroid arthropathy.

The volume of synovial fluid in normal knee joint may vary from 0.5 to 5.0ml, and in inflamed knee it can go up to 100ml[48,49]. Based on prior investigations, we set the volume of synovial fluid in affected knee joint at the time of intra-articular injection, at 2 to 3ml which remained in the joint after aspiration of effusion. The injection volume for study medication contains a mixture of corticosteroid, local anesthesia and some saline dilution was set at 2-3 ml. For ease of calculation, the final volume in the knee joint cavity after intra-articular injection was set at 5ml. If one vial (40mg, 1ml) of TA is injected, we estimated the concentration of triamcinolone was 8mg/ml. Likewise, when one ampoule (5mg, 1ml) of DE was injected, the concentration of DE was estimated as 1mg/ml.

Our original study doses were selected based on results published by Fubini et al[50]. Fubini reported that the chondrocyte count in cartilages from normal horse and bovine joint dropped by methylprednisolone within the range of 0.3-0.4 mg/ml to 1.0-4.0mg/ml. Murphy et al[39]. found methylprednisolone at concentrations lower than 0.01 mg/ml did not depress proteoglycan synthesis in normal equine cartilage explants but concentration of 10mg/ml methylprednisolone severely depressed it and failed to recover even after methylprednisolone removal.

Initially, we planned to compare the chondrotoxicity of two agents when injected from one vial (8mg/ml vs. 1mg/ml), half vial (4mg/ml vs. 0.5mg/ml) as usual dosage, and one sixteenth vial (0.5mg/ml vs. 0.0625mg/ml) as the minimal concentration of triamcinolone that is supposed to have definite chondrotoxicity. Our pilot study showed complete inhibition of chondrocyte proliferation in all groups of TA. Only when after filtering the particles of TA suspensions, which is differ from in vivo state, the inhibition of chondrocyte proliferation was not noticed in TA 0.5 mg/ml group. Thus, we had to decrease the study concentrations and find

a lower TA concentration which inhibits the cell proliferation, then compared the results of corresponding DE concentration which is same volume in the consistent commercial packages (8:1).

In this study, TA (suspension) showed definite inhibition of chondrocyte proliferation at 31.25 μg/ml (1/256 vial) and higher concentrations. But DE (solution) has not revealed the limitation of cell proliferation from 1/2560 to 1/16 of ampoule while the chondrocyte proliferation was inhibited in 0.5 mg/ml (1/2 ampoule). IL-1β stimulation did not alter the proliferation of chondrocyte in each concentration of drugs. Fahey M. et al[51]. also have described that DE had no effect on cell viability at any of the concentrations (1-100nM) tested in their investigation in porcine chondrocytes.

From the differences of DE, (1) the shorter exposure to articular cartilage (rapid absortion), (2) lesser inhibition of chondrocyte proliferation during the same exposure time; we can infer that DE is a safer agent than TA for intra-articular injection.

If the benefit effect of TA and DE is similar, the drug which has lower chondrotoxicity on articular cartilage could be the better choice for intra-articular injection. Even if triamcinolone acetate was more effective than dexamethasone disodium phosphate, the relatively low chondrotoxicity and faster absorption of DE should be considered by clinicians. Therefore, using DE, a more frequent intra-articular injection schedule (shorter interval than 3 months of ACR guideline) could be attempted if necessary. Also, if ultra low concentrations (somewhere between 1/2 ampoule and 1/16 ampoule) of DE has some clinical benefit, then shorter interval of intra-articular injection could be a new treatment strategy. Derendorf H et al[46]. demonstrated that betamethasone phosphate showed peak plasma concentration during the first hour after IA injection while betamethasone acetate absorbed slower from joint into blood stream for up to 2 weeks. Similarly, regarding the rapid absorption of dexamethasone disodium phosphate, the dexamethasone acetate could

be the proper agent for this shorter interval treatment strategy. Since acetate formula could lengthen the duration of action of dexamethasone in the joint than phosphate formula. Further clinical and in vitro studies are needed to identify the efficacy and safety of multiple dexamethasone injection in low concentration.

Since the steroid-induced arthropathy represents progressive degradation of cartilage even after the repeated intra-articular injections has been cancelled, the apoptosis and necrosis of corticosteroid treated chondrocytes is a matter of interest. Nakazawa et al[52]. implanted the human articular cartilage into the back of severe combined immunodeficiency (SCID) mice. One month later, six weekly injection of corticosteroid in the subcutaneous cavity around the graft cartilage in SCID mice was treated. They demonstrated increasing chondrocyte apoptosis in the SCID/hu model, but were not able to found statistically significant difference among the corticosteroids, though dexamethasone acetate was most apparent to induce the chondrocyte apoptosis. Hossain et al[53]. showed that significant suppression of chondrocytes proliferation and induction of apoptosis occurred with concentration 0.1-50 μg/ml of DE compared with untreated cells. But, in our study, neither apoptosis nor necrosis was detected in group TA4 , TA7, DE4 and DE7 as well as control groups. Aigner T et al[54]. reported that chondrocyte apoptosis is not a widespread phenomenon in normal aging and osteoarthritic knee of human. Reason of our unpredicted result is unknown. But we suspect that the inhibition of chondrocyte proliferation by corticosteroid might be a mechanism of cell cycle arrest.

This study showed that the proportion of G2/M cells increased in corticosteroid treated groups; even in the groups they did not inhibit proliferation of chondrocyte (TA7, DE4, DE7). In condition where proliferation of chondrocyte is inhibited (Group TA4), G2/M cell level increased significantly and this changing of distribution in cell cycle may play a role in corticosteroid chondrotoxicity. The anti-proliferative effects of glucocorticoids in fibroblast[55], osteoblast[56] and epithelial

origin cells[57] have been reported as result of G1 cell cycle arrest. Where Fouty et al[58] found DE stimulate G1-S phase transition in asthmatic fibroblast. But to date, there are very few reports published regarding the description of cell cycle in steroid induced chondrotoxicity.

Since the specimens we used were removed from most normally looking surface of the severe osteoarthritis patients, it could be a limitation of our study. Even though Lafeber et al[59]. reported that grossly nonfibrillated human articular cartilage is more vital than fibrillated cartilage, it is comparable biochemically and histologically to normal cartilage and its use is preferable to animal cartilage. On the other hand, because IA injection is usually used in osteoarthritis where the articular destruction is not severe enough to need a TKR, this cartilage could bear more similarity with conditions we are interested in. We compared the corticosteroids under clinical basis, by volume of the agents (ml), since physicians usually do not calculate the molar mass and mole of the steroids in their practice, whereas the concentration of TA and DE are almost constant among the products throughout the worlds.

V. CONCLUSION

Dexamethasone disodium phosphate(DE) has lesser chondrotoxicity than triamcinolone acetate and it could be considered as a safer corticosteroid for frequent repeated intra-articular injection. A more frequent intra-articular injection of low concentrations of dexamethasone might be a viable new treatment strategy for osteoarthritic knees unless the low concentration does not have the desired treatment effect.

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ABSTRACT (IN KOREAN)

관절내 스테로이드 주사의 인간 관절연골에 대한 세포독성

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서론: 무릎 관절의 퇴행성 관절염에서 관절강내 스테로이드 주사가 활액막에 작용하여 관절액 과다생성 억제, 부종 및 동통의 감소를 일으켜 치료 효과를 나타낸다. 하지만 관절강내에 주입된 스테로이드가 활액막외에 관절연골에도 동시에 노출되므로 이것으로 인한 관절연골 세포의 세포독성이 문제되고 있고 1년내에 네차례 이상의 스테로이드 주사치료를 제한하고 있다. 본 연구의 목적은 관절강내 주사용으로 사용할 수 있는 triamcinolone acetate와 dexamethasone disodium phosphate의 관절 연골에 대한 세포독성을 조사하고 비교하는 것이다.

재료 및 방법: 퇴행성 관절염으로 인공슬관절 전치환술을 시행받은 환자 10명의 관절연골을 배양하여 여러 농도의 TA와 DE에 접촉시켜 3일동안의 세포증식을 MTT방법을 이용하여 검사하고 그 결과를 대조군(배지 및 0.9%식염수)과 비교하였다. 특정 농도의 TA군과 DE군 그리고 대조군에서 2일간의 약물노출 후 apoptosis와 cell cycle에 대해 각각 FACS방법을 사용하여 조사하였다. Glycosaminoglycan(GAG) 성분량를 특정 농도의 TA군과 DE군 그리고 대조군에서 2일간의 약물노출 후 측광법으로 측정하였다. 10ng/ml IL-1β를 함께 처리한 연골세포에서 상기 세포 증식, 세포 주기 및 GAG 성분량 측정을 각각 검사하였다. 모든 표본에서 세차례 반복 실험하였고 통계처리는

Student's t-test를 사용하였으며 유의수준은 0.05이하로 정하였다.
결과: 31.25 μg/ml보다 높은 농도의 TA군 (TA1-TA5)에서 연골세포의 증식이 약물에 노출된 3일동안 억제되었다 ($p<0.02$). 모든 DE군은 3일동안 연골세포 증식에 억제를 일으키지 않았으며 이중의 가장 고농도가 DE1군 (62.5 μg/ml)이고 이 농도는 1mg/1ml를 함유한 DE에서 1/16앰플을 무릎 관절내에 주사하여 얻어지는 관절내의 약물 농도로 추정된다. 또한 0.5mg/ml(1/2앰플)의 DE에서는 연골세포의 증식억제가 나타났다. GAG 성분량은 62.5 μg/ml의 농도를 갖은 TA4군에서 현저하게 감소하였고 ($p=0.003$), DE군과 TA7군 (3.125 μg/ml)에서는 GAG 성분량의 감소가 없었다. 연골세포의 증식억제를 일으켰던 TA4군과 정상 세포 증식을 보인 TA7군 모두에서 apoptosis를 관찰하지 못 했다. 10ng/ml IL-1β를 처리한 실험에서는 모든 TA군에서 연골세포의 증식이 억제되었다 ($p<0.02$). 10ng/ml IL-1β를 함께 처리한 후 GAG 성분량을 측정한 결과, 모든 TA군에서 대조군에 비해 의미있는 감소($p<0.05$)를 나타냈으며, 이중 triamcinolone acetate의 농도가 낮아질수록 GAG 성분량의 감소가 줄어들었고 TA5군 (31.25 μg/ml)에서 가장 많은 양이 측정되었으며 그 이하의 농도에서는 다시 GAG 성분량이 TA의 농도 감소에 따라 줄었다. 모든 DE군에서도 GAG 성분량이 대조군에 비해 의미있게 줄었으며 ($p<0.05$) 그 양은 TA5군과 비슷한 수준을 유지하였다.
결론: 관절연골에 대한 세포독성을 고려한 측면에서 dexamethasone disodium phosphate이 triamcinolone acetonide보다 관절강내 주사치료의 목적으로 반복 사용하기에 안전한 약물이다. 또한 퇴행성 관절염의 무릎에 대해서 증세 회복을 유지하기 위해 낮은 농도의 dexamethasone disodium phosphate를 기존보다 더 빈번하게 관절강내 주사를 시행하는 방법을 고려할 수 있다.

핵심되는 말 : chondrotoxicity, triamcinolone acetate, dexamethason disodium phosphate, intra-articular injection, knee