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Triamcinolone acetonide reduces viability, induces oxidative stress, and alters gene expressions of human chondrocytes

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Abstract. – OBJECTIVE: Patients with knee osteoarthritis (OA) are sometimes prescribed intra-articular injections of glucocorticoids (GCs), such as triamcinolone acetonide (TA). Whether GCs cause chondrotoxicity is not known. We wished to ascertain if TA induces toxicity by causing oxidative stress and alters expression of P21, growth differentiation factor (GDF)15, and cFos.

PATIENTS AND METHODS: Primary chondrocytes isolated from 10 OA patients undergoing total knee replacement surgery were incubated with TA (0, 1, 5, 10 mg/ml), with or without 100 μM vitamin C for 7 and 14 days for viability assays and 48 h for oxidative stress and gene expression analyses.

RESULTS: TA significantly decreased chondrocyte viability and increased the ratio of oxidized glutathione to total glutathione suggesting an increase in oxidative stress. Vitamin C significantly increased the viability and decreased the oxidative stress of cells treated with 5 mg/ml TA. Expression of *P21*, *GDF15*, and *cFos* increased significantly when TA was added (5.17 ± 2.4-, 4.96 ± 3.1-fold for *P21*, 9.97 ± 2.9- and 4.2 ± 1.6-fold for *GDF15*, and 6.65 ± 4.8-, 12.96 ± 8.3-fold for *cFos* at 1, and 5 mg/ml TA, respectively).

CONCLUSIONS: TA induced chondrotoxicity by increasing oxidative stress and altering expressions of genes involved in cell death. The addition of vitamin C decreased oxidative stress and increased viability, suggesting that antioxidants might attenuate TA toxicity in cartilage.

Key Words:
Triamcinolone acetonide, Oxidative stress, Chondrocyte, Gene expression.

Introduction

Osteoarthritis (OA) of the knee is one of the most common types of arthritis[1], and several treatment options are available[2]. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in patients with joint inflammation owing to their lower toxicity compared with corticosteroid-based drugs. However, some patients are allergic or resistant to the effects of NSAIDs. Ultimately, anti-inflammatory drugs such as synthetic glucocorticoids (GCs) are employed by intra-articular injection into affected joints[2,3]. These drugs, in complex with their glucocorticoid receptor (GC-receptor), act as transcription factors that inhibit expression of pro-inflammatory proteins by interfering with the activity of the transcription factor nuclear factor-kappa B, or enhancing expression of anti-inflammatory proteins directly[4].

Triamcinolone acetonide (TA) is a GC drug used commonly for joint injection in OA patients owing to its extended duration of effect[5]. However, regular use of TA and other GCs is not recommended due to increased risks of joint infection[6]. The balance between its treatment effects and adverse effects on cartilage and chondrocytes is not completely understood[2,5]. Results from several studies have shown contradictory results regarding the benefits *versus* toxic effects of TA and or other drugs of the same class. Concerning the protective effects, a study in patients with rheumatoid arthritis reported that a TA derivative, triamcinolone hexacetonide, decreased release of collagen oligomeric matrix protein from cartilage, which in turn lowered cartilage degradation[7]. However, several studies have shown the toxic effects of TA and other GCs on chondrocytes, cartilage, and tissues around the joint, both *in vitro* and *in vivo*[8,9]. A study in OA chondrocytes treated with

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betamethasone or prednisolone resulted in an increase in the number of necrotic chondrocytes[10]. Furthermore, a study in primary tenocyte-like cells from the shoulder joint capsule showed that TA decreased cell viability in a dose- and time-dependent manner, and that repeated administrations of TA caused permanent damage to cells[11].

Exact mechanisms of TA-induced chondrotoxicity are incompletely understood. One mechanism could be a result of the oxidative stress caused by TA. This notion was supported by a study in a retinal cell line that showed a significant increase in levels of reactive oxygen species (ROS) inside TA-treated cells compared with dexamethasone-treated and non-treated cells[12]. However, that result contradicted another study in retinal pigment epithelial cells which reported that TA increased levels of reduced glutathione (GSH), suggesting a decrease in oxidative stress[13].

Other than reacting with cellular macromolecules, ROS produced in cells also alter expressions of genes involved in cell cycle checkpoint and apoptosis. As a result, two p53-target genes, *P21* and g*rowth differentiation factor* (*GDF*)*-15*, were of interest owing to their association with cell cycle control and their signature response to oxidative stress[14]. Another gene of interest was *cFos* because it is a subunit of a transcription factor involved in response to oxidative stress: activator protein-1 (AP-1), whose target genes are involved in cell proliferation and apoptosis[15].

We aimed to assess the cytotoxicity and oxidative stress caused by TA on primary chondrocytes from OA knees. The effect of the antioxidant ascorbic acid in relieving TA toxicity upon primary chondrocytes was determined, as well as expression of genes related to cell cycle control and response to oxidative stress.

Patients and Methods

Ethical Approval of the Study Protocol

The study protocol was approved by the Human Ethics Committee of Thammasat University (MTU-ED-OT-4-099/57; Pathumthani, Thailand). All participants were given full information regarding the study objectives and potential problems. All patients provided written informed consent.

Sample Collection

Articular cartilage samples were collected from 10 patients (40-70 years old), diagnosed with Kellgren-Lawrence grade 3-4 radiographic OA of the knee and who underwent knee replacement surgery at the Department of Orthopaedic Surgery, Faculty of Medicine, Thammasat University Hospital.

Isolation of Primary Chondrocytes

Cartilage samples were subjected to several steps of enzyme digestions according to a modified version of Goldring's method[16], except for an overnight incubation time with 1% filtered collagenase (Gibco, Billings, MT, USA) at 37°C instead of 4 h. Released chondrocytes were filtered through a Cell Strainer (diameter, 70 μm) and centrifuged at 1,000 × *g* for 5 min. Cell pellets were resuspended in culture medium [DMEM/F12 with 10% fetal bovine serum and a 1% antibiotic-antimycotic mixture (Gibco)] and cultured until they reached confluence. Chondrocytes in passage 1 isolated from each patient were used in all conditions tested.

Viability Assay

Chondrocytes were seeded into 96-well plates at 15,000 cells per well and cultured for 48 h. Then, 100 μl of 0, 1, 5, and 10 mg/ml TA (LBS. Laboratory, Bangkok, Thailand) diluted with the culture medium was added to each well in triplicate per condition, and incubated for 7 and 14 days. Concentrations were adjusted from clinically used TA concentrations (40 mg/ml)[17] according to the ratio between the mean surface area of knee articular cartilage and surface area of the plates[9,18]. After the incubation was complete, TA was discarded, and 90 μL of fresh media was added. Cell viability was determined by incubation with 10 μL of PrestoBlue® (Invitrogen, Carlsbad, CA, USA), then measuring absorbance at 540 nm with a microplate spectrophotometer. Viabilities of TA-treated cells were calculated by dividing optical density (OD) of the treated cells by OD of their matching controls. For antioxidant treatments, 100 μM of vitamin C (ascorbic acid) (VESCO, Bangkok, Thailand) was added to the culture medium. The vitamin C concentration was chosen according to Yudoh's study[19]. In addition, the effects of various vitamin C concentrations (10-1000 μM) were determined in our preliminary experiments. The results showed that vitamin C at 100 μM gave the highest viability percentage.

GSH Assay

Chondrocytes were seeded into six-well plates at 10^6 cells per well and cultured for 48 h. Then, 0, 1, and 5 mg/ml of TA with and without 100 µM of vitamin C were added to each well in duplicates per condition and incubated for 48 h. Then, cells were collected and lysed for protein collection by the mechanical disruption method using syringe needles. GSH levels were measured using an OxiSelect™ Total Glutathione Assay kit (Cell Biolabs, San Diego, CA, USA).

Gene Expression

Cell treatments were the same as those for the GSH assay except that vitamin C was not added. RNA was extracted using an RNeasy® Mini kit (Qiagen, Stanford, VA, USA). Total RNA (500 ng) was used for cDNA synthesis using AccuPower® RT Premix (Bioneer, Seoul, South Korea). Real-time PCR was carried out using AccuPower® 2 GreenStar™ qPCR Master Mix (Bioneer). Expression was normalized with *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*)[20]. All primer pairs for real-time PCR except for *P21* gene were designed with NCBI Primer-Blast[21] and are shown in Table I. One primer of each primer pairs binds to the exon-exon region of the genes; therefore, the primers would not amplify genomic DNA. Efficiencies of real-time PCR conditions were between 97-102% (r^2 = 0.97–0.99). Comparative quantification cycle ($2^{-\Delta\Delta Cq}$) method was used for calculation of relative gene expression[22].

Statistical Analysis

Analysis of variance with homogeneity of variance test and Tukey's *post hoc* honest significant difference correlation was used to analyze differences between treatment groups using SPSS v-13.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

Results

TA Reduced Chondrocyte Viability

Results from the viability assay showed a decrease in viability of TA-treated chondrocytes in a dose-dependent manner. Differences of viabilities between the two incubation periods were not significant. Viabilities of cells treated with 1, 5, and 10 mg/ml TA are shown in Table II. Differences in viability compared with controls were significant at all concentrations tested ($p < 0.01$), and were significantly different between 1 and 10 mg/ml of TA ($p = 0.037$). Vitamin C slightly increased the viability of cells treated with 1 mg/ml TA with no statistical significance. This increase was significant only for in cells treated with 5 mg/ml TA at day 14 (Table II).

TA Induced Oxidative Stress in Chondrocytes

The ratio of oxidized glutathione to total glutathione (GSSG:total GSH) was used to assess the oxidative stress effect of TA on chondrocytes[23]. Durations of the incubations times and TA concentrations that could induce stress without killing chondrocytes were based on results from the viability assay. Value of GSSG:total GSH in TA-treated chondrocytes increased in a dose-dependent manner (0.087 ± 0.025, 0.394 ± 0.12, and 0.517 ± 0.135 in cells treated with 0, 1, and 5 mg/ml TA, respectively) with a significant difference at 5 mg/ml TA ($p = 0.02$) (Figure 1). Results suggested that cells underwent oxidative stress as a result of TA treatment. Addition of vitamin C decreased the value of GSSG:total GSH (0.089 ± 0.023, 0.129 ± 0.048, and 0.123 ± 0.031 in cells treated with 0, 1, and 5 mg/ml TA, respectively) with a significant difference when cells were treated with 5 mg/ml TA ($p = 0.025$).

Table I. Primers and annealing temperature for real-time PCR.

Genes	Primers (5′-3′)	Tm (°C)	Amplicon size (bp)
P21[42]	Forward – TGGAGACTCTCAGGGTCGAA	61.8	123
	Reverse – CCAGGACTGCAGGCTTCC	60.5	
GDF15	Forward – AAACGCTACGAGGACCTGCT	59.3	242
	Reverse – GTCACGTCCCACGACCTTGA	61.4	
cFos	Forward – ACTTCATTCCCACGGTCACT	57.3	277
	Reverse – GCAGCCATCTTATTCCTTTCC	57.9	
GAPDH	Forward – GAAGGTGAAGGTCGGAGTC	57.2	237
	Reverse – GAAGATGGTGATGGGATTTC	53.7	

Table II. Viability of TA-treated chondrocytes compared with their matching non-treated controls (% viability ± standard error; SE) with or without vitamin C at 7 days and 14 days of incubation (n = 10).

Days of incubation	Treatment	1 mg/ml TA		5 mg/ml TA		10 mg/ml TA	
		% Viability	*p*	% Viability	*p*	% Viability	*p*
7	TA	75.43 ± 2.5	0.8	70.10 ± 2.9	0.15	66.07 ± 2.4	0.28
	TA with vitamin C	76.27 ± 3.0		75.58 ± 2.03		61.34 ± 2.5	
14	TA	78.30 ± 4.3	0.28	68.55 ± 3.7	0.035*	66.05 ± 2.7	0.81
	TA with vitamin C	80.81 ± 4.4		76.10 ± 3.2		65.22 ± 3.15	

*Statistical significance between viabilities of cells treated with and without vitamin C.

TA Affected Expressions of Genes Related to Regulation of the Cell Cycle and Response to Oxidative Stress in Chondrocytes

When chondrocytes were treated with TA, expressions of genes involved in regulation of the cell cycle (*P21*) and response to oxidative stress (*GDF15*, *cFos*) increased significantly compared with those of non-treated chondrocytes (Figure 2). Differences in mRNA levels between cells treated with 1 and 5 mg/ml TA were not significant in any of the three genes. No significant differences ($p = 0.4$) in mRNA levels between cells treated with 1 and 5 mg/ml TA were observed. Compared with non-treated controls, levels of mRNA from *P21* increased 5.17 ± 0.8- and 4.2 ± 0.5-fold ($p \leq 0.001$), those of *cFos* increased 4.96 ± 1.0- ($p = 0.029$) and 6.65 ± 1.5-fold ($p < 0.01$), and those of *GDF15* increased 9.97 ± 0.9- and 12.97 ± 2.6-fold ($p \leq 0.001$) in cells treated with 1 and 5 mg/ml TA, respectively.

Discussions

TA is a widely used GC for treating OA patients by intra-articular injection despite potential

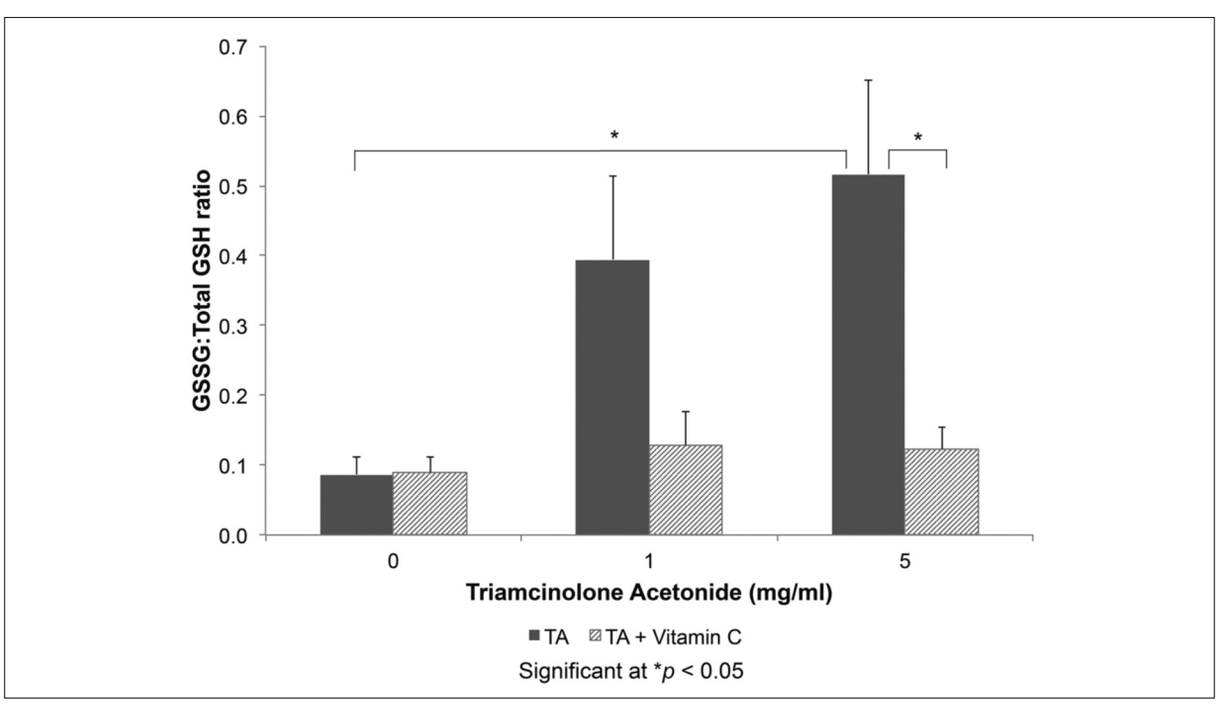

Figure 1. Ratio of oxidized glutathione to total glutathione with SE in TA-treated chondrocytes with or without vitamin C (n =10).

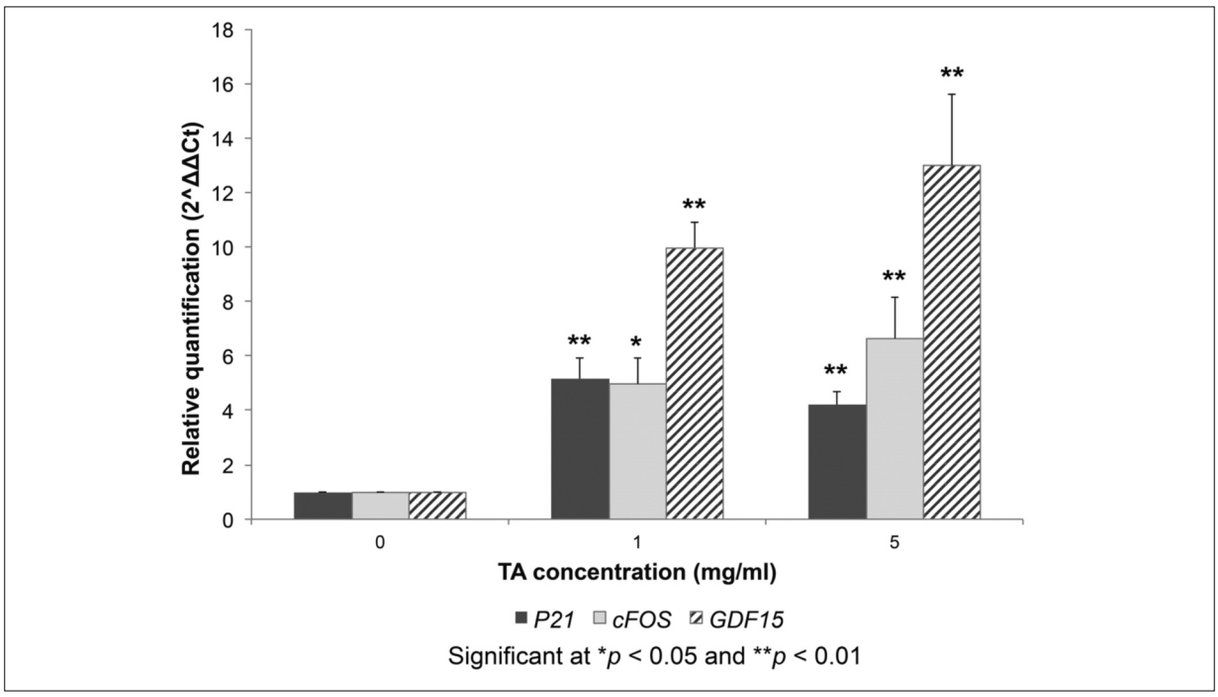

Figure 2. Relative mRNA level with SE of *P21*, cFos, and *GDF15* in chondrocytes treated with TA compared with non-treated controls. GAPDH was used as an internal control (n = 10).

side effects to the injected joints. Several studies have reported that GCs can decrease cartilage integrity and chondrocyte viability. However, the cause of chondrotoxicity is not known.

Although chondrocytes in early stage of OA resulted in increasing cell proliferation, chondrocytes used in this study were taken from patients with late-stage OA, which are vulnerable to apoptosis[24]. We showed that one of its mechanisms of action was to inflict oxidative stress upon chondrocytes. Most studies of the effects if GCs have been done in chondrocyte cell lines or healthy animals, neither of which represents the disease condition in OA patients. We used primary chondrocytes from the articular cartilage of OA patients, which had pathological conditions similar to those of cells in patients undergoing TA injection.

Results from this study suggested that TA decreases chondrocyte viability and increases oxidative stress to chondrocytes in a dose-dependent manner. They were consistent with results from Farkas et al[10] and Braun et al[9] which demonstrated that GCs increased chondrocyte death. Results from Zhao et al[25] also reported that dexamethasone increased autophagy of chondrocytes leading to a decrease in viability. Moreover, our results were consistent with those of Chung et al[12] which reported TA-induced ROS production in retinal cells. How TA induces oxidative stress in chondrocytes is not known. There have been reports on the effects of dexamethasone and corticosterone in decreasing antioxidant enzymes and mitochondrial activities in different cell types, such as hippocampal neurons[26] and rat pheochromocytoma cell line[27]. Other studies in lung endothelial cells and neural cells reported that dexamethasone altered expression of nitric oxide synthase[28] and genes of endogenous antioxidant enzymes[29]. Taken together, these data could explain how TA induced oxidative stress in chondrocytes in the present study.

The addition of vitamin C decreased oxidative stress significantly upon treatment with 5 mg/ml TA, which is consistent with the results from the viability assay. This might be explained by results from a study demonstrated that dexamethasone increased expression of *SVCT2*, a vitamin C transporter, in rabbit intervertebral disc cells[30]. Therefore, the higher benefits of vitamin C in chondrocytes treated with higher concentration of TA might be from the higher expression of SDVT2, hence more vitamin C was transported into chondrocytes. Moreover, vitamin C might

affect chondrocytes proliferation via other pathways independent of its antioxidant role as it is an important cofactor of several enzymes[31]. Results from this study suggested that vitamin C might be a potential supplement used for reducing TA-induced oxidative stress in chondrocytes of OA patients. It might be locally co-injected into the joints as nutritive mixture solution[32], or as sodium ascorbate in deoxygenated saline[33]. Using vitamin C in combination with other antioxidants (e.g. vitamin E) might also increase antioxidation capacity and cell viability[34].

Alteration in expression of genes involved in cell cycle progress and apoptosis by oxidative stress was evaluated in our work. Results from gene expression analyses showed that all three genes had a significant increase in expressions compared with those of controls. One of the deleterious consequences of oxidative stress is DNA damage. Hence, the increase in *P21* expression observed in our study could have been due to induction of a p53-dependent mechanism that occurs in response to DNA damage, which leads to the arrest of cell-cycle progression[35]. *GDF15* is a target gene of p53 rapidly induced by stresses. Increased levels of *GDF15* in several investigations suggest its roles in both preventions of apoptosis[36] and induction of cell death[37]. The increased expression of *cFos* observed in the present report could have resulted from ROS activation of mitogen-activated protein kinase signaling pathways that can prevent[38,39] and induce cell death[15]. We suspect that the increased expression of *GDF15* and *cFos* observed in our study had a destructive effect upon chondrocytes because of a significant decrease in cell viability after TA treatment.

Limitation of this paper was that chondrocytes were cultured in monolayer, while, in the body, they have a three dimensional interaction with neighboring cells and are embedded in the extracellular matrix. Chondrocytes used in all experiments were in passage 1 in order to minimize phenotypic changes. Studies reported that chondrocytes at passage 2 cultured for less than 21 days could maintain their chondrocyte-specific protein expressions[40,41].

Conclusions

We demonstrated that TA was associated with chondrotoxicity by increasing oxidative stress and led to increased expressions of genes involved in cell cycle check-point and apoptosis. Use of antioxidants in combination with GCs might decrease destructive effects in cartilage. Further, *in vivo* studies may help to ascertain this suggestion.

Declaration of Funding Interests

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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