

Biochemical and Histological Evaluations of Articular Cartilages Preserved in Cold Storage Solution Containing Green Tea Catechin, EGCG

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Abstract : Although epigallocatechin-3-*O*-gallate(EGCG), a major poly phenolic constituent of green tea, has various pharmacological and biological activities including anti-carcinogenic, anti-thrombotic and anti-inflammatory effects, relatively a little is known about its beneficial effects on the non-frozen preservation of mammalian cells and tissues. In this study, articular cartilages from human knee joint were pretreated with 1 mM EGCG for 1 d and then preserved in serum-free RPMI 1640 media with 1% antibiotic-antimycotic solution at 4°C for 1, 2 and 4 wk. After cold preservation, chondrocyte viability(CCK-8 assay), biochemical and immunohistochemical composition[glycosaminoglycans and(type II)collagen], and biomechanical property(compressive elastic modulus) were assessed, respectively. Chondrocyte viability of cartilages pretreated with EGCG was significantly well-maintained for at least 2 wk with high contents of glycosaminoglycan and total collagen. These beneficial effects of EGCG pretreatment were more confirmed by histological and immunohistochemical observations showing well-preserved cartilaginous structures and delayed denaturation of the extracellular matrices in preserved specimens. The compressive elastic modulus(MPa) of cartilages pretreated with EGCG was well-maintained as much as that of fresh specimens without any increase as the progress in the preservation period. Here were also found that fluorescein isothiocyanate-conjugated EGCG were widely distributed through the matrix and clearly observed at the chondrocytes in the lacunas. Taking these results into consideration, it is suggested that EGCG may play an effective role in preserving articular cartilages, which be exploited to craft strategies for the long-term preservation of osteochondral allografts under cold storage conditions.

Key words: *Epigallocatechin-3-O-gallate, cold preservation, articular cartilages, chondrocyte viability, compressive elastic modulus*

1. Introduction

Articular cartilage injury is challenging to treat because of limited intrinsic healing capacity.^{1,2} Although the natural history of isolated cartilage lesions still is unknown,³ it generally is thought that articular cartilage injury may predispose the involved joint to accelerated degeneration.^{2,4} This problem is magnified by the relative frequency of cartilage injuries.^{5,6} The unsuitability of total joint replacements for young, active individuals has provided the stimulus to search for alternative treatments in the field of biologic resurfacing of

joints. Numerous surgical treatments for chondral and osteochondral lesions have been described, but each method has certain limitations.^{7,8} On the other hand, tissue engineering techniques and regenerative medicine have been explored as a potential method to restore natural tissue and repair lesions.^{9,10} Nevertheless, no optimal method for the cryopreservation of mammalian tissue or organ as well as tissue engineered medical products and has been established.¹¹ Also, current methods can result in a substantial loss of function and lead to damage and destruction of the cells and tissues.

The number of tissue or organ transplants has increased substantially in recent years with the advances in the surgical methods and the development of immunosuppressive agents.^{12,13} Ideally, tissues should be transplanted immediately

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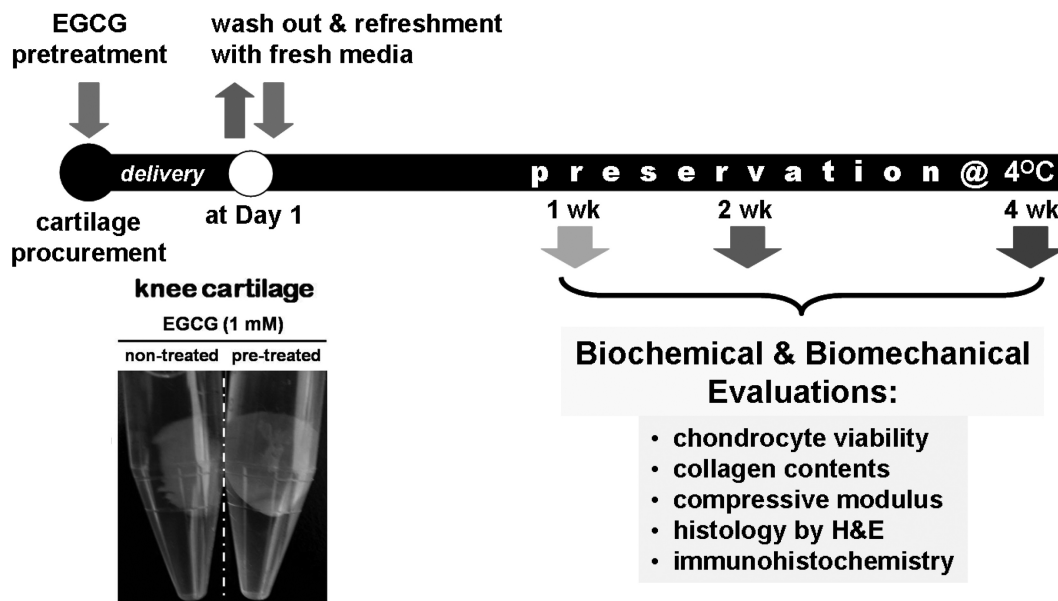


Figure 1. Experimental scheme of this study. Cartilage specimens were procured, pretreated without or with 1 mM EGCG in serum-free RPMI 1640 media with 1% antibiotic-antimycotic solution, and kept at 4°C. The specimens were then delivered to the senior author (Prof Hyon) within 1 day of procurement. Fresh cartilages were also delivered in the media with 10% FBS and 1% antibiotic-antimycotic solution at room temperature. Immediately after receiving, the cartilage specimens were replaced with 20 mL of fresh media without FBS and then stored at 4°C for 1, 2 and 4 wk without changing the storage solution. After preservation, biochemical, immunohistochemical and biomechanical analyses were performed as described in Materials and Methods.

from the donor to the recipient. However, this is not always possible, and the problem of tissue preservation is very important for ensuring a successful transplantation. Therefore, it is essential to develop storage solutions that can maintain the viability and function of the tissues or organs for longer periods. Usually, a dysfunction of the transplants occurs as the result of free radicals due to ischemia, which triggers lipid peroxidation of the cell membrane when the blood flow is restarted.^{14,15} Particularly, ischemic osteonecrosis can produce a permanent residual deformity of the immature femoral head and epiphyseal cartilage damage.¹⁶ A good storage solution should prevent this peroxy lipid generation. Since this is related to cell proliferation and division, longer term tissue preservation for transplantation would become possible if cellular metabolism can be controlled.¹⁷

From this point of view, our attention has been paid to (–)-epigallocatechin-3-O-gallate (EGCG), the predominant catechin from tea, since it has a wide range of pharmacological activities, including antioxidant, anticancer, anti-proliferative, anti-inflammatory and anti-thrombotic effects.^{18–20} Different from these biological activities of EGCG, its cytopreservative effects on mammalian cells and tissues were examined in order to design a cell- or tissue-preserving medium/solution at

physiological temperature in our previous studies.^{18–20} The present study provided support to a scenario in which EGCG might play a key role in preserving articular cartilages by maintaining chondrocyte viability and metabolism in cartilages as well as matrix structure. Therefore, EGCG pretreatment can be exploited to craft strategies for the long-term preservation of osteochondral allografts under cold storage conditions.

2. Materials and Methods

2.1 Cartilage Specimen Collection and Cold Preservation

Human articular cartilages were obtained from knee joints of 10 outpatients (male or female, 58–86 years old, 15–20 mm in diameter and 2–2.5 mm in thickness) undergoing total knee arthroplastic surgery at Marunouchi Hospital, Nagano, Matsumoto-si, Japan. As shown in Fig 1, these specimens were procured under sterile conditions from the donor and placed in a storage solution [serum-free RPMI 1640 media (Sigma-Aldrich Co, St Louis, MO) with 1% antibiotic-antimycotic solution (including 10,000 units penicillin, 10 mg streptomycin and 25 mg amphotericin B per mL, Sigma-Aldrich Co)] with 1 mM EGCG (Teavigo™, DSM Nutritional Products Ltd, Basel,

Switzerland),²¹ and kept at 4°C (EGCG pretreatment). The specimens were then delivered to the senior author (Prof Hyon) within 1 d of procurement. Fresh cartilages (control) were delivered in the media with 10% fetal bovine serum (FBS, Sigma-Aldrich Co) and 1% antibiotic-antimycotic solution immediately after procurement from the donor at room temperature. Because of this necessary processing delay between tissue procurement from the donor and its delivery, 1 day was set as the data point for the fresh specimen.

On receipt of the specimens, cartilage tissues were replaced with 20 mL of fresh media without FBS and then stored at 4°C for 1, 2 and 4°C wk without changing the storage solution. At the end of each preservation period, biochemical, histological, immunohistochemical and biomechanical analyses were performed as described below. All procedures involving human subjects received prior approval from Marunouchi Hospital, Osaka City University Graduate School of Medicine and the Institutional Review Board of Institute for Frontier Medical Sciences, Kyoto University, and all subjects providing written informed consent.

2.2 Chondrocyte Viability

The number of viable cells was quantified indirectly using a highly water soluble tetrazolium salt [WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitro-phenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (Dojindo Lab, Kumamoto, Japan), reduced to formazan dye by mitochondrial dehydrogenase. The viability of chondrocytes was found to be directly proportional to the metabolic reaction products obtained in WST-8. According to the manufacturer's instructions, cartilage specimens following cold preservation were incubated with WST-8 in the last 4 h of the incubation period (24 h) at 37°C in the dark. Fresh cartilages were regarded as their own controls, respectively. The absorbance was determined at 450 nm in an ELISA reader (Spectra Max 340, Molecular Device Co, Sunnyvale, CA).

2.3 Glycosaminoglycan (GAG) and Collagen Contents

GAG content was determined by using a dimethylmethylene blue (DMMB) dye binding assay kit (Blyscan kit, Biocolor Ltd, Newtownabbey, Northern Ireland, UK) according to the manufacturer's instructions. Chondroitin sulfate, provided with the kit, was used as the GAG standard. Briefly, 50 mg of cartilage was homogenized and solubilized. A 100 mL aliquot from each homogenate was added to 1 mL DMMB, in duplicate, and mixed periodically at 25°C for 30 min. Solutions

were then centrifuged, and the supernatant discarded. The remaining pellet was suspended in 1 mL of the provided dissociation reagent, and absorbance measurements were taken at 656 nm. The determined GAG contents were normalized with the chondrocyte viability.

Total cartilage collagen levels were determined by using a Sircol collagen dye binding assay kit (Biocolor Ltd) according to the manufacturer's instructions. Briefly, 50 mg of cartilage was homogenized, and total acid pepsin-soluble collagens were extracted overnight using 5 mg/mL pepsin in 500 mL of 0.5 M acetic acid. One mL of Sircol dye reagent was added to 100 mL of each homogenate, in duplicate, and incubated at 25°C for 30 min. After centrifugation, the pellet was suspended in 1 mL of alkali reagent, and absorbance was read at 540 nm. The determined collagen contents were normalized with the chondrocyte viability.

2.4 Histological and Immunohistochemical Analyses

At the completion of the predetermined preservation, each cartilage specimen was rinsed with phosphate-buffered saline (PBS, pH 7.4) and immediately fixed with 2% glutaraldehyde, 2% paraformaldehyde and 0.2% CaCl₂, followed by embedding in paraffin. The tissue blocks were sectioned by a 5 mm thickness, stained with hematoxylin and eosin (H&E, for general evaluation) and Safranin-O/fast green (for GAG content and distribution), and immunostained with rabbit monoclonal antibody against type II collagen. The prepared sections were examined using an optical/fluorescence microscope (Biozero-8000, Keyence, Osaka, Japan).

Additionally, the cartilage specimens were pretreated with 1 mM fluorescein isothiocyanate (FITC, Sigma-Aldrich Co)-conjugated EGCG (FITC-EGCG) in a storage solution for 1 d and preserved for 2 wk at 4°C in order to determine whether EGCG penetrated into the matrix of cartilages with further incorporation into the cytosol of cells in lacunae or not. The tissue sections were prepared as mentioned above and then observed under a fluorescence microscope (Keyence).

2.5 Biomechanical Analysis

At each data point, cartilage specimens were removed from the storage solution and placed immediately in a -80 freezer until biomechanical testing could be done. At the time of testing, each specimen was thawed at room temperature (25°C) in PBS plus proteinase inhibitors. A cartilage compression disk with an intact articular surface measuring 10 mm in diameter × 1 mm in thickness was made from each specimen. To determine

the compressive elastic modulus, this cartilage disk was then subjected to dynamic visco-elastic compression (Rheogel E-4000, UBM Co Ltd, Japan) with 0.5% strain. All data were collected at a frequency of 10Hz.

2.6 Statistics

All variables were tested in three independent storages for each experiment, and each experiment was repeated twice ($n=6$). Quantitative data were expressed as mean \pm standard deviation (SD). Statistical comparisons were carried out with a one-way analysis of variance (ANOVA, SAS Institute Inc, Cary, NC, USA), which was followed by the Bonferroni test for the multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1 Protective Effects of EGCG on Chondrocyte Viability of Cartilages

Preserving the cartilage specimens without EGCG pretreatment resulted in a significant ($p < 0.05$) time-dependent reduction in the chondrocyte viability (Fig 2). Their viability was reduced to approximately 39% already after 1 wk and 30% after 2 wk. When the specimens were pretreated with 1 mM EGCG, their viability loss was only 31% even after 2 wk, suggesting that EGCG pretreatment prevents the reduction of cellular viability. This compound seemed to actively penetrate into the cartilaginous layer, leading to the protection of cell viability. Although this decrease in the viability was accelerated with the progress in the incubation time, there was a

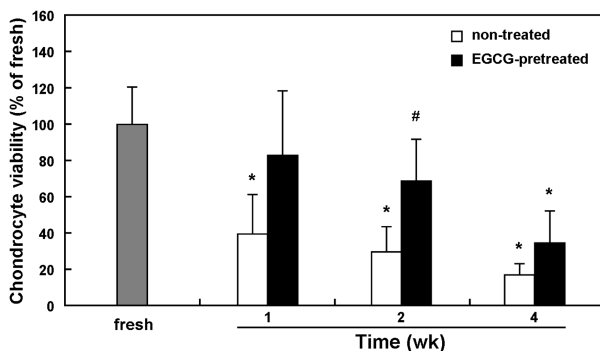
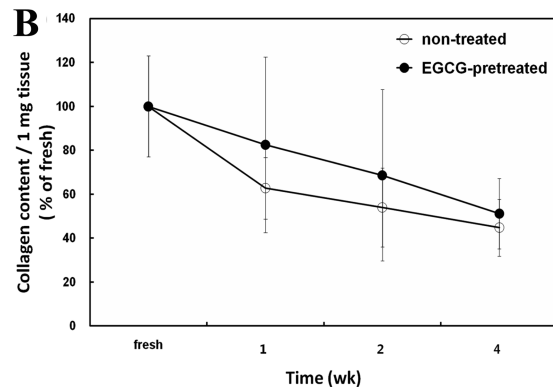
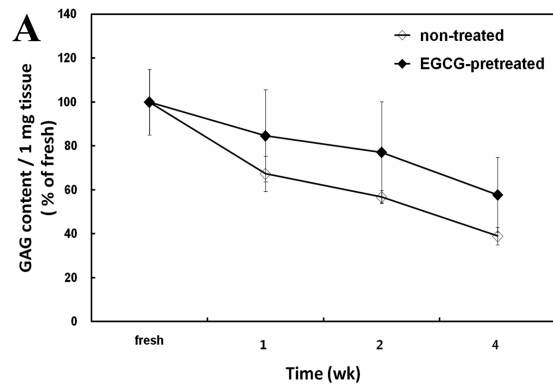


Figure 2. Chondrocyte viability in cartilages pretreated without or with EGCG. Cell viability was measured with a WST-8 assay as described in Materials and Methods. The results are reported as mean \pm SD ($n=6$) and analyzed by the Bonferroni test (* $p < 0.05$ vs fresh control and # $p < 0.05$ vs with EGCG at each time point).

significant ($p < 0.05$) difference between cartilages pretreated without and with EGCG after 2 wk. However, after 4 wk, the protective activity was relatively less exerted by EGCG, and the viability was reduced to about 44%.

3.2 Protective Effects of EGCG on Contents of GAG and Collagen in Cartilages

GAG and collagen contents of the cartilages correlated well with their viability as shown in Fig 3. After 1 wk of preservation without EGCG pretreatment, GAG content was about 67%, which was significantly ($p < 0.05$) lower than that of the fresh specimen (Fig 3A). As the progress in the preservation time, this declined to 57% at 2 wk and then decreased dramatically to 39% at 4 wk. However, pretreatment with EGCG improved the chondrocyte metabolism in cartilages. After 2 wk of preservation with EGCG pretreatment, GAG content was measured as approximately 77%, which was appreciably higher than that of preservation without EGCG pretreatment. After 4 wk of storage with EGCG, the content significantly ($p < 0.05$) decreased to 58% compared with the fresh. However, this was still noticeably higher than that of storage without EGCG for the same duration. Collagen contents showed a similar pattern to that of GAG. Although there was no significant difference in the contents between preservation without and



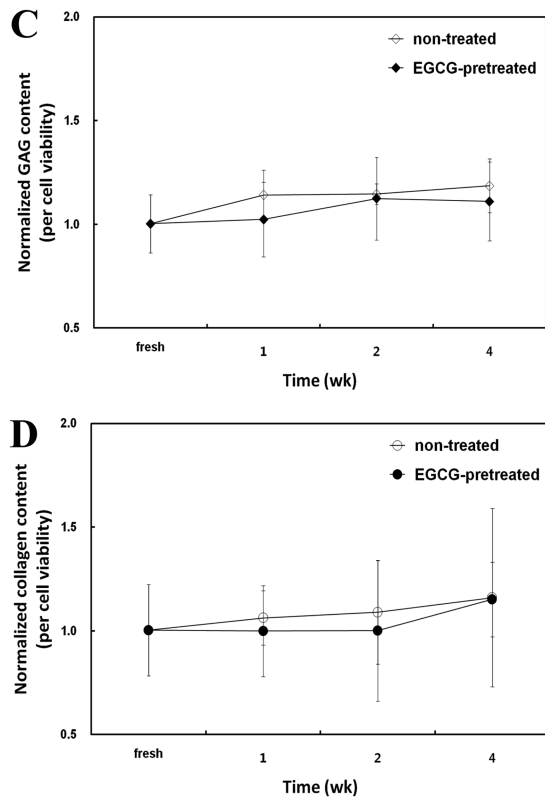


Figure 3. ECM composition of cartilages pretreated without or with EGCG. GAG contents(A) and total cartilage collagen levels(B) were determined by using a DMMB dye binding assay kit and a Sircol collagen dye binding assay kit, respectively, as described in Materials and Methods. Additionally, the normalized contents(per cell viability) of GAG(C) and total collagen(D) were plotted. The results are reported as mean±SD(n=6) and analyzed by the Bonferroni test(* $p < 0.05$ vs. fresh control).

with EGCG pretreatment, the values in EGCG-pretreated cartilages were remarkably higher than those in non-treated specimens(Fig 3B). In addition, it was revealed that the extracellular matrix(ECM) components of the cartilages did not change appreciably during the study period(Fig 3C and 3D). After storage without and with EGCG pretreatment, there essentially were no fluctuation in the normalized contents(per cell viability) of GAG and total collagen.

3.3 Protective Effect of EGCG on Matrix Structure of Cartilages

Routine histological and immunohistochemical examinations were performed on cartilage specimens preserved without or with EGCG(Fig 4). Through the H & E staining, it was difficult to differentiate between cartilages preserved without and with EGCG(Fig 4A). However, there was a numerical trend favoring EGCG-pretreated specimens

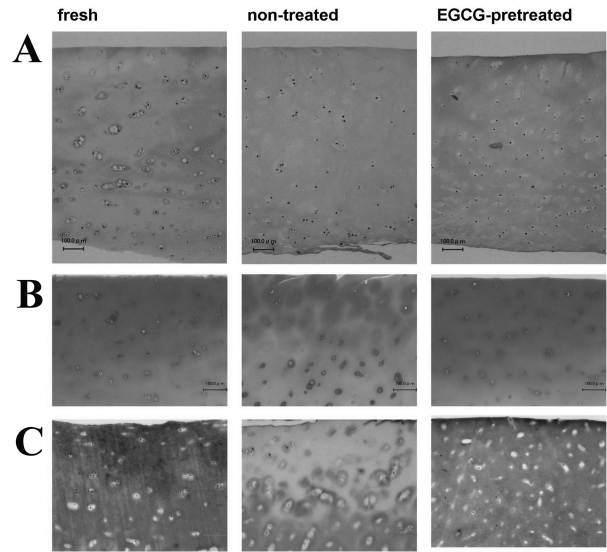


Figure 4. ECM structure of cartilages pretreated without or with EGCG. Cartilage specimens were stained with either H&E(A) or Safranin-O/fast green(B, for GAG content and distribution), and immunostained with rabbit monoclonal antibody against type II collagen(C). The photographs shown in this figure are representative of 6 independent experiments, showing similar results.

preserved for 2 wk. EGCG-pretreated specimens stained positively with Safranin-O showing abundant presence of GAG, which was comparable to that of fresh ones(Fig 4B). In contrast, non-treatment resulted in an appreciable decrease in the positivity for Safranin-O. The peripheral region of each cartilage had a higher cell density and stained more intensely for GAG in comparison to the central region. The cells in the lacunas of fresh and EGCG-pretreated cartilages were well-maintained with an original chondrocytic phenotype compared with those of non-treated specimens. Immunohistochemical examination using type II collagen antibody showed the presence of this macromolecule in the cartilages pretreated with EGCG and again, the cell density and staining intensity were greatest in the periphery of the specimens(Fig 4C). In each of the specimens examined, type II collagen primarily was found intracellularly. Comparison of cartilage tissues that stained positive for both GAG and type II collagen within the same specimen revealed that Safranin-O positive tissues had the usual association with type II collagen typically seen in normal or fresh articular cartilage.

3.4 Penetration and Incorporation of FITC-EGCG into Cartilages

The penetration of EGCG into the matrices of cartilages and its incorporation into the cytosol of cells were confirmed after

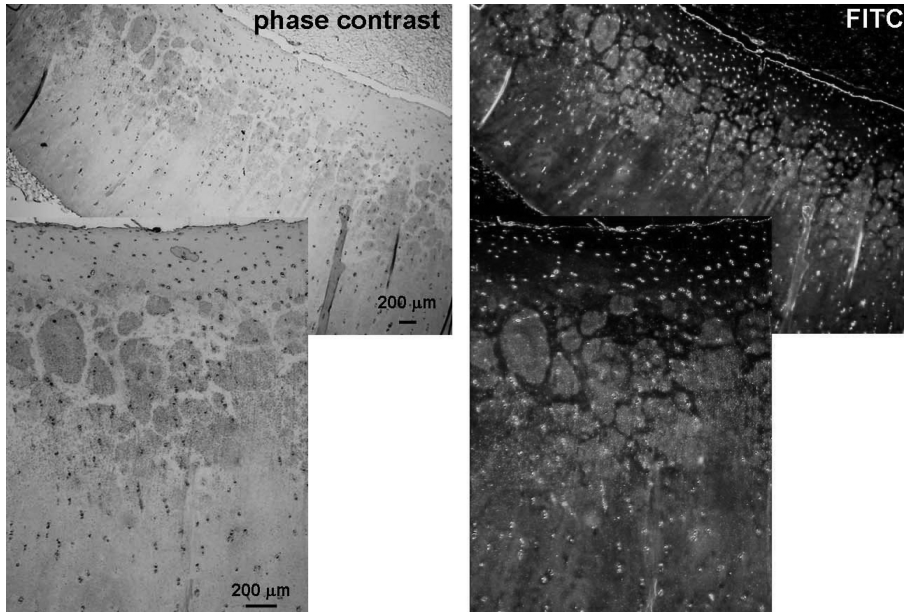


Figure 5. Penetration of EGCG into cartilaginous matrices and its intracellular incorporation. Cartilage specimens were pretreated with 400 mg/mL FITC-EGCG, preserved for 2 wk and then observed under a fluorescence microscope. The micrographs shown in this figure are representative of 6 independent experiments, showing similar results.

2 wk of storage with FITC-EGCG treatment(Fig 5). FITC-EGCG was located at any places through the matrix and clearly observed at the cells in the lacunas of the cartilages treated with FITC-EGCG. These results imply that although the cellular sensitivity and response to EGCG would be different from those of FITC-EGCG, EGCG could easily bind to the matrices as well as the cells and be incorporated into the cytosol, which would lead to protection of the cells from oxidative stress generated during cold preservation period.

3.5 Protective Effect of EGCG on Biomechanical Property of Cartilages

Biomechanical testing evaluated the function of the existing cartilage matrix, and opposite to the contents of GAG and collagen, the strength of non-treated cartilages appreciably increased with time(Fig 6). The value significantly($p<0.05$) increased after 2 wk, which was about 2.0 times higher than that of the fresh specimens. However, no significant changes in the compressive elastic modulus, if any, slightly increased, were detected in EGCG-pretreated cartilages.

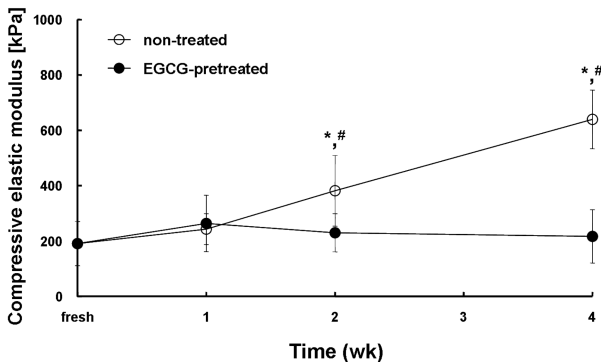


Figure 6. Biomechanical property of cartilages pretreated without or with EGCG. Compressive elastic modulus was determined by dynamic visco-elastic compression as described in Materials and Methods. The results are reported as mean±SD(n=6) and analyzed by the Bonferroni test(* $p<0.05$ vs. fresh control and # $p<0.05$ vs. with EGCG at each time point).

4. Discussion

It was found that chondrocyte viability of EGCG-pretreated cartilages was significantly well-maintained for at least 2 wk with high contents of GAG and total collagen, histological and immunohistochemical appearance of well-preserved cartilaginous structures, delayed denaturation of ECM, and no alterations of compressive elastic modulus. These results can be attributed to the fact that EGCG might actively penetrate into the cartilaginous layer and protect the chondrocyte viability and metabolism in cartilages as well as the matrix structure.

Histologically, metachromatic staining of cartilage matrix preserved without EGCG for 2 wk was lost mainly from perichondrocyte area, suggesting that chondrocytes which lost their viability digested matrix by themselves. It was found that

EGCG penetrated into the matrices of cartilages and was incorporated into the cytosol of cells, enabling cartilaginous structures of preserved cartilages to be well-preserved and allowing denaturation of ECM to be delayed. Because the viability of chondrocytes in cartilages was kept high at least 2 wk, this procedure may be effective not only in preserving the allogeneic osteochondral graft but also chondrocyte transplantation, such as autologous chondrocyte implantation and allogeneic chondrocyte transplantation. In case of chondrocyte transplantation, we have to harvest cartilage and carry it to cell processing center, which takes a few hours to a couple of days. Because maintaining cell viability is highly important in cell transplantation, this procedure may be useful in carrying cartilage.

A point to be solved is that there was a time-dependent decline in the metabolic activity (ECM contents) of the cartilage during cold preservation. These findings correlated highly with chondrocyte viability. In contrast, a recent study showed that storage of human osteochondral allografts in serum-free culture media at 4°C provided significantly better preservation of the metabolic activity and biomechanical property of the tissues with no significant detrimental alterations after 2 wk of storage.²² When normalized with the chondrocyte viability of the tissue, however, the contents of GAG and total collagen in the present study remained stable throughout the 4 wk study period. There are a number of explanations for these inconsistencies, including the determination methods of cell viability and ECM contents as well as the measurement method of compressive elastic modulus. We have to further investigate the limit of storage time using *in vivo* experiment.

In the present study, the hypothesis was testified that EGCG pretreatment enables to extend storage duration for the cold preservation of human articular cartilages from knee joints. To our knowledge, little has been published on the beneficial effects of EGCG on the prolonged cold storage of human osteochondral grafts and the mechanism at cellular level. The penetration of EGCG into the matrices of cartilages and its cellular uptake into chondrocytes in the lacunas were clearly observed. It is not dangerous to conclude that these phenomena could be related to the intrinsic characteristics of polyphenolic compound, EGCG. It has already been known that this compound, due to its amphipathic properties, easily binds to ECM, lipid membranes and any types of intracellular proteins.²³⁻²⁵ Therefore, it seems that this compound enables to be internalized into cell cytosol and further to be translocated into nucleus, which leads to modulating the exogenous signals

directed to genes required for the survival and apoptosis of cells. Although exact mechanism of the internalization of EGCG into cells had not been elucidated yet, some evidence was reported showing that EGCG was bound to the membrane as well as incorporated into the cytosol and the nucleus of cancer cell lines, such as PC-9 and HT-29 cells.^{26,27} Consistently, recent study has demonstrated that expression of the metastasis-associated 67 kDa laminin receptor might confer EGCG responsiveness to cancer cells at physiologically relevant concentrations, suggesting that the gallate moiety of EGCG may be critical for receptor binding and subsequent activity.^{28,29} Moreover, the observation that nucleic acids extracted from catechin-treated cells were colored implied that because both galloyl and catechol groups of EGCG were essential for DNA binding, both groups seemed to hold strands of DNA via their branching structure.³⁰

Taking these results into consideration, it is concluded that EGCG plays effective roles in preserving articular cartilages by penetrating into the matrices of cartilages and incorporating into the cytosol of chondrocytes, which is regarded as a feasible action mechanism of EGCG. Thus, it is suggested that these results can be exploited to craft strategies for the long-term preservation of osteochondral allografts under cold storage conditions.

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