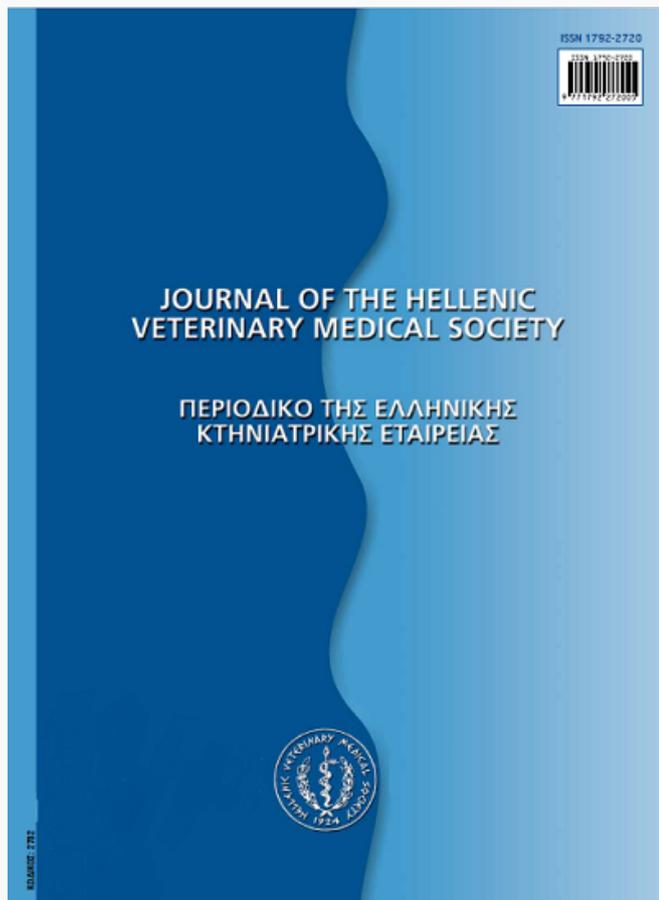


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G. HONG, Y. HWANG, G. KIM

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## **Cytotoxicity of Contrast Media Iohexol on IL-1beta stimulated Bovine Chondrocytes**

**Hong G.<sup>1</sup>, Hwang Y.<sup>1</sup>, Kim G.<sup>1\*</sup>**

<sup>1</sup>*Laboratory of Veterinary Surgery, College of Veterinary Medicine, Chungbuk National University, Chungbuk 28644, South Korea*

**ABSTRACT.** The aim of this study was to determine the cytotoxic and metabolic effects of iohexol on cultured bovine chondrocytes in clinical dose. Chondrocytes were exposed to 50%, 25% and 12.5% iohexol and 50% mannitol for 2 hours. Cell proliferation, apoptosis and necrosis were analyzed. Real time PCR was performed for aggrecan, collagen type I and II gene expression. Cells in alginate beads stimulated by interleukin-1 $\beta$  (IL-1 $\beta$ ) were analyzed for cytotoxicity. MTT assay showed that 50% iohexol inhibited the proliferation of cells at 2 hours culture period. Propidium iodide results showed significantly higher dead cells at 50% iohexol compared to control, however PCR results revealed that chondrogenic gene were not affected. Cells in alginate beads stimulated with IL-1 $\beta$  showed significantly higher percentage of dead cells at 50% iohexol exposure ( $p < 0.05$ ). These results suggested that iohexol has a cytotoxicity on chondrocytes and this cytotoxic effect possibly increased in inflammatory joint diseases.

**Keywords:** Alginate beads, Chondrocytes, Chondrogenic gene, Cytotoxicity, Iohexol

*Corresponding Author:*

Gonhyung Kim, DVM, PhD  
Address: Laboratory of Veterinary Surgery, College of Veterinary Medicine  
Chungbuk National University  
Chungdae-ro 1, Seowon-Gu, Cheongju  
Chungbuk 28644, South Korea  
E-mail: ghkim@cbu.ac.kr

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## INTRODUCTION

The diagnostic imaging technology among various animal diagnostic methods makes it possible to examine the internal organs in a non-invasive manner, thereby making it available as an important diagnostic method. In order to uplift the efficiency of evaluation in imaging diagnosis, contrast media were developed and applied to the gastrointestinal tract, the urinary system, blood vessels, pancreas and biliary duct and joint. While CT and X-ray utilizing a contrast medium are applied to examine the overall shape of joint cartilage, the degree of destruction, ligaments and so forth less frequently than MRI system in the joints, still they are widely used in the veterinary medicine in particular (De Rycke et al., 2015; Hong et al., 2010; Van Vynckt et al., 2013).

Iodized contrast media for CT and X-ray diagnosing are divided largely into ionic contrast media and non-ionic contrast media, and chemically into monomer and dimer. Side effects are also reported on the cardiovascular system, neurological system, renal system such as vascular endothelial cell damage, blood clots, hypotension, hypersensitive reaction and shocks, while the common contrast media for clinical use are evaluated as highly safe (Bettmann et al., 1997; Katayama et al., 1990; Margulies et al., 1991; Nyman et al., 1980). High osmotic pressure and chemical toxicity of contrast media can also play an important role in causing cellular toxicity (Heinrich et al., 2005; Wang et al., 1998; Zhang et al., 2000). However, the pathogenesis of contrast media-induced side effects is not clearly understood.

The contrast medium injected into the joint is quickly absorbed into the blood vessel through the synovial tissue and excreted through the kidney, and is not observed in the joint after 3 hours of injection (Edwards et al., 2007). Iohexol, a non-ionic contrast medium having low osmotic pressure, shows a high glomerulus filtration rate and is a safe contrast medium with little direct side effects on the tissue and cells, compared with other iodized contrast media, thus typically making it available for use in both humans and animals in clinical fields (Barkin et al., 1991). However, it is reported that iohexol with known safety is toxic for vascular endothelium cells (Takatsuki et al., 2004; Zhang et al., 2000) and renal cells (Duan et al., 2006; Gong et al., 2010; Lee et

al., 2006). While numerous reports on the effects of iohexol on several types of cells are made available recently, research reports on its effects on chondrocytes are not to be found in the medical area as well as in the veterinary science. Therefore, this study will try to elucidate the effect of iohexol on cellular toxicity and metabolism in clinically applicable concentrations with bovine chondrocytes.

## MATERIALS AND METHODS

### Isolation and culture of bovine chondrocytes, and measurement of osmotic pressures

Bovine chondrocytes were isolated from six-month-old Holstein calf that died accidentally, without suffering from any osteoarthritic disease. Full thickness articular cartilages were aseptically collected from the distal femoral condyle and digested with collagenase type I (0.1%) (Welgene, Daegu, South Korea) for 18 h at 37°C in a shaking water bath. After isolation, the cells were cultured with high glucose DMEM with phenol red (Dulbecco's modified eagle's medium; Welgene, Daegu, South Korea) containing 10% FBS (Fetal bovine serum; Welgene, Daegu, South Korea) at 37°C in 95% air and 5% CO<sub>2</sub> humidified atmosphere. Culture medium was changed two times per week. After the cells were confluent, they were trypsinized with 0.25% trypsin-EDTA (Welgene, Daegu, South Korea) and used for this study. The cellular survival rate was evaluated with Trypan blue staining during cell culture. Osmotic pressures of Iohexol (Omnipaque 300; GE Healthcare, Cork, Ireland) and mannitol (Daehan D-Mannitol 20%; Daehan Pharm Co., Korea) were measured by using the micro-osmometer.

### MTT assay

To measure the proliferation of chondrocytes, an MTT assay was conducted. Bovine chondrocytes were set at  $2\sim 4 \times 10^5$  cells/ml, added to 96well plate 100  $\mu$ l each and cultured in the incubator at 37°C and 5% CO<sub>2</sub>. After they were cultured for 24 hours, medium was changed into isotonic saline, 50%, 25%, 12.5% iohexol and a 50% mannitol and incubated for 2 hours. The medium were removed, and all of the wells were added with 100  $\mu$ l of DMEM without phenol red (Welgene, Daegu, South Korea) and 10  $\mu$ l of MTT

solution (final concentration of MTT: 0.5 mg/ml). After 2 hours incubation, the 96-well plate was emptied and 50  $\mu$ l of dimethyl sulfoxide (DMSO; Junsei Chemical Co. Ltd, Japan) added to each well to dissolve the formazan crystals. The optical density (OD) was measured at 540 nm using a spectrophotometer (Emax, Molecular Devices, Sunnyvale, CA, USA). The optical densities were converted into percentages using the following formula: Cell viability (%) = (OD of test/ OD of control-isotonic saline) x 100. This process was repeated 4 times.

### **Double stain of propidium iodide and hoechst 33258**

Bovine chondrocytes were set at  $2\sim 4 \times 10^5$  cells/ml, cultured in 6 well plate at 37°C and 5% CO<sub>2</sub>. After they were cultured for 24 hours, medium was replaced with 50%, 25%, and 12.5% iohexol and a 50% mannitol diluted from commercial solution and cultured for 2 hours. The cells were trypsinized for 5 minutes with 0.25% Trypsin-EDTA and separated, cautiously mixed with the final concentration set at 5  $\mu$ g/ml Hoechst 33258 (H33258; Sigma, St. Louis, MO, USA), and cultured for 15 minutes. To observe the necrotic cells, 20  $\mu$ g/ml Propidium Iodide (PI) was added to the mixture, and observed with a UV filter of a fluorescent microscope. More than 200 cells were observed from various angles and results were documented.

### **RNA extraction and real time PCR**

For reverse transcription PCR (RT-PCR), total RNA was extracted from the  $1\sim 2 \times 10^5$  cells using easy-blue (iNtRON, Seoul, Korea) and first-strand cDNA was synthesized by Maxime RT Premix Kit (iNtRON, Seoul, Korea). Primers which were designed previously to detect specific mRNA are described in Tabel 1. The PCR conditons were set for one minute at 95°C, for 5 seconds at 95°C, for 15 seconds at 55°C. The PCR products were electrophoresed on 1% agarose gel and stained with ethidium bromide. The amount of cDNA was measured by reactions of 1  $\mu$ l cDNA template with 10  $\mu$ l of 2 x SYBR premix Ex Taq (TaKaRa Bio Inc.) and 10 pmol of the specific primers. Forty cycles reaction was carried out at 95°C for 15 seconds, 58°C for 15 seconds, and 72°C for 30 seconds. The amount of

product was measured of the intensity of fluorescence and the mRNA expression level of target genes was normalized by  $\beta$ -actin.

### **Three-dimensional culture of chondrocytes in alginate beads**

The effect of iohexol on inflammation chondrocytes were evaluated with alginate beads culture. Chondrocytes and alginate solution were prepared at a final concentration of  $5 \times 10^6$  cells/ml and 1.2% alginic acid sodium salt. A bead-shaped alginate gel was completed in 102 mM calcium chloride solution by using 200  $\mu$ l pipette tips. After washing beads, those were cultured for 2 weeks in 10% FBS (Welgene, Daegu, South Korea) containing DMEM. The inflammation cell model was cultured in DMEM with 10 ng/ml recombinant canine interleukin 1- $\beta$  (IL-1 $\beta$ ; R&D Systems, Abingdon, UK) for 24 hours. Beads were divided into 4 groups and were cultured; 1) culture in normal medium, 2) culture in 50% iohexol for 2 hours, 3) culture in IL-1 $\beta$  for 24 h, 4) culture in IL-1 $\beta$  for 24 hours and then 50% iohexol for 2 hours. An evaluation of cell toxicity was conducted utilizing a double dying method of PI and H33258 three times.

### **STATISTICAL ANALYSIS**

Evaluation of the cell proliferation rate and the results of a real-time PCR were expressed in an average  $\pm$  deviation, each value being compared and evaluated by using one-way analysis of variance (ANOVA) and post hoc Dunnett test. In case the P value was less than 0.05, it was considered as statistically significant.

## **RESULTS**

### **Osmotic pressures**

In evaluating the effects of the contrast medium on chondrocytes, mannitol was selected for comparisons of high osmotic pressures to evaluate the presence or absence of the effects of osmotic pressures of the contrast medium itself on the cells. The osmotic pressure of the undiluted solution of the contrast medium reached 885 mOsm/kg and the undiluted solution of mannitol pointed to 1013 mOsm/kg. The 50% mannitol (828 mOsm/kg) selected for the com-

**Table 1.** Primer sequences used in RT-PCR and Real time PCR.

Gene	Primer sequence	Product size (bp)	Accession number
Collagen type I	F 5'TGCTGGCCAACCATGCCTCT	120	AB008683
	R 5'CGACATCATTGGATCCTTGCA		
Collagen type II	F 5'ATCCATTGCAAACCCAAAGG	147	X02420
	R 5'CCAGTTCAGGTCTCTTAGAG		
Aggrecan	F 5'CACTGTTACCGCCACTTCCC	303	U76615
	R 5'GACATCGTTCCACTCGCCCT		
$\beta$ -Actin	F 5'CGCACCCTGGCATTGTCAT	227	K00622
	R 5'TCCAAGGCGACGTAGCAGAG		

parative group showed an osmotic pressure higher than any other iohexal concentration which is lower than 50% iohexol (592 mOsm/kg).

### Cell proliferation

After stimulating cells by isotonic saline solution, 50%, 25%, 12.5% iohexal and 50% mannitol for 2 hours, differences were observed under the microscope. While the cells cultured in saline solution maintained the way they grew on the normal medium, it was observed that those cells stimulated by 25% iohexol for 2 hours declined in the cell proliferation depending on concentration. The cells stimulated by 50% iohexal were not clearly observed due to the inability of microscopes to focus due to the contrast medium properties. The MTT results showed outcomes similar to those observed under the microscope. The results of MTT assay showed that 50%

iohexol inhibited significantly the proliferation of bovine chondrocytes at 2 hours culturing ( $p=0.030$ ). The cell viability stood at  $85.9\pm 5.6$  at 50% iohexol,  $91.8\pm 4.6$  at 25% iohexal,  $95.1\pm 3.9$  at 12.5% iohexal, and  $94.9\pm 3.3$  at 50% mannitol (Figure 1).

### Apoptosis of chondrocytes

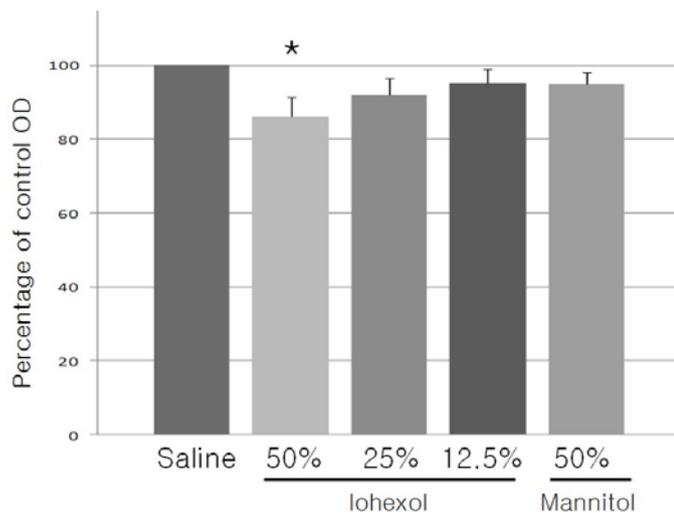
The dead cells was observable since the nucleus was dyed in the red by PI, and apoptosis was also observable since the nucleus was dyed in the blue by H33258, and it was confirmed through the observation of the nucleus with respect to whether it is condensed or made amorphous (Figure 2). The number of dead cells dyed by PI was observed as significantly high in the 50% iohexol group, compared with isotonic saline solution ( $p<0.05$ ). The dead cell rate was pointed to  $17.9\pm 12.3$  in 50% iohexol,  $7.8\pm 3.8$  in 25% iohexol,  $6.3\pm 3.9$  in 12.5% iohexal,  $5.6\pm 2.6$  in 50%

**Table 2.** The percentage of dead cells stained with propidium iodide.

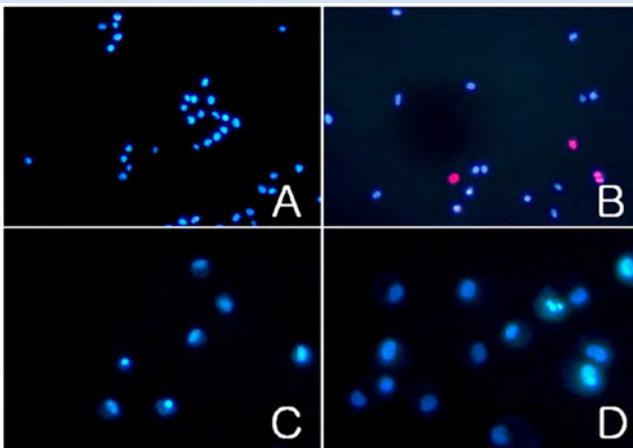
Isotonic saline	Iohexol			Mannitol
	50%	25%	12.5%	50%
2.0 $\pm$ 0.6	17.9 $\pm$ 12.3*	7.8 $\pm$ 3.8	6.3 $\pm$ 3.9	5.6 $\pm$ 2.6

Data are reported as means  $\pm$  SD. \* The percentage of dead cells stained with propidium iodide is significantly higher at 50% iohexol compared to isotonic saline group ( $p<0.05$ )

**Fig 1.** MTT results expressed as a percentage of control absorbance. The results of MTT assay showed that 50% iohexol inhibited significantly the proliferation of bovine chondrocytes at 2 hours culturing ( $p=0.030$ ).



**Fig 2.** Dead cells stained with propidium iodide are observed more frequently in 50% iohexol (B) than saline (A). No significant iohexol-induced apoptotic nuclei are not observed in hoechst stained cells for 2 hours, however those are observed in cells at 12 hours culturing (C, D).



mannitol and  $2.0 \pm 0.6$  in saline solution, respectively (Table 2). With respect to the observation of apoptotic nucleus showing abnormal forms of nucleus such as condensation, collapse due to contrast media, they were not frequently observed in all the groups during 2 hours culture period. While numerous apoptotic nucleus were observed in the cells stimulated by the contrast medium for 12 hours.

### Comparison of gene expression

To evaluate the effects of exposure to the contrast medium on the metabolism of cells, RT-PCR with respect to mRNAs of collagen type II and Aggrecan, particular indicators of chondrocytes, and mRNA of Collagen type I, a degenerative indicator of chondrocytes, was conducted. All the genes amplified by compounded primer were observed in expected molecular weight in PCR conducted 35 times. It was confirmed that in the cells cultured used for this experiment, mRNAs of Collagen type II and Aggrecan were strongly manifested normally. The results of evaluations of the effects of iohexol stimulation on the quantity of the chondrogenic genes through a quantitatively real-time PCR three times repetitively show that collagen type I and II increased and Aggrecan decreased, while no significant differences were observed (Figure 3).

### Cytotoxicity evaluation of cells stimulated by IL-1 $\beta$

Unlike monolayer culture, cells were cultured with their original form maintained in alginate beads, and proliferated cells were observed as the culturing time elapsed. When IL-1 $\beta$  was stimulated for 24 hours at a 10 ng/ml concentration, cell proliferation was observed to be effectively suppressed. It was observed that 15.4% of the dead cells were dyed on PI when the cells were stimulated in a 50% iohexol, compared with the control group where about 7.9% of the cells were dyed. Stimulation by IL-1 $\beta$  for 24 hours caused 21.6% cell death. However stimulation by a 50% iohexal for 2 hours after that by IL-1 $\beta$  for 24 hours caused significantly higher percent of dead cells (31.3%) compared to those of other experimental conditions ( $p < 0.05$ ) (Table 3).

### DISCUSSION

Noticeable strides have been made in the diagnostic imaging with the aid of image technology through the application of contrast media in the veterinary science as well as human medical science. The use of contrast media, a non-invasive approach, made it possible to conduct precise diagnoses in internal and surgical medicine. It is true that X-ray and CT are less frequently used for joint cartilage disorders, rather

**Table 3.** The effect of 50% iohexol on IL-1 $\beta$  stimulated chondrocytes cultured in alginate beads.

Control	50% Iohexol	10 ng/ml IL-1 $\beta$	10 ng/ml IL-1 $\beta$ + 50% Iohexol
7.9 $\pm$ 4.3	15.4 $\pm$ 7.1	21.6 $\pm$ 6.3	31.3 $\pm$ 6.6*

Data are reported as means  $\pm$  SD of the percent of dead cells. Cells were cultured with 10 ng/ml IL-1 $\beta$  for 24 hours and/or 50% Iohexol for 2 hours. \* The percentage of dead cells stained with propidium iodide is significantly higher at 10 ng/ml IL-1 $\beta$  + 50% iohexol group compared to control group ( $p < 0.05$ ).

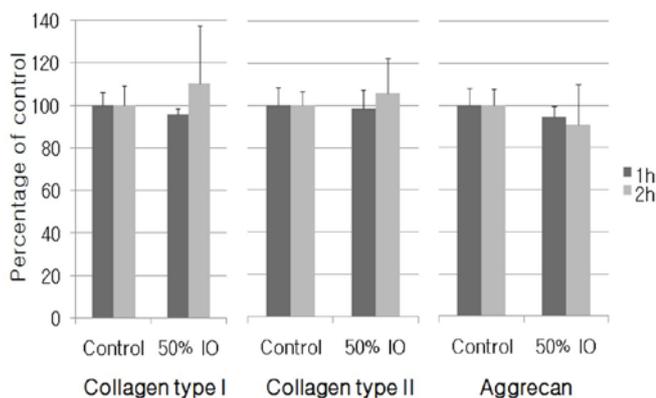
than new diagnostic approaches by means of image technology such ultrasonography and MRI technology. However, where such new approaches are hardly available, X-ray and CT approaches are generally applied. Therefore, the development of safer contrast media is steadily under way.

This study evaluated the effects of iohexol generally used in conducting arthrography on the chondrocytes of joint cartilage. It was confirmed that iohexol causes chondrocytes death. It has been reported that iodinated contrast media can induce apoptosis of endothelial cells even with brief exposure of 15 minutes (Zhang et al., 2000). Iohexol was also found to cause apoptosis of neutrophils following a short exposure (Fanning et al., 2002). Much of the contrast medium in the joint was absorbed through the synovia within 3 hours of injection (Edwards et al., 2007). Therefore a toxicity

evaluation of long-time stimulation to the cells was not conducted in this study, because it is clinically insignificant to evaluate it. Inhibitory effects on cell growth are seen when exposed to iohexol having a low osmotic pressure compared with mannitol with high osmotic pressure. Minor inhibitory effect of osmotic pressure on chondrocytes was observed here. The effects of contrast media on the metabolism of cells were confirmed by means of molecular biological techniques. This experiment indicates that, during the stimulation for 2 hours, a time span when the contrast medium exists clinically in the joint, any effect of iohexol on the cell metabolism was hardly observed.

It is reported that substances inducing inflammation factors such as IL-1 $\beta$ , IL-6 and TGF- $\alpha$  that are secreted in abundance in synovial cells of animals suffering from arthritis affect the proliferation and metabolism of chondrocytes (Blanco et al., 1995; Fiorito et al., 2005; Zhou et al., 2008). To evaluate the toxicity of contrast media for chondrocytes in the patients with arthritis, model cells inducing inflammation by stimulating the cells cultured on a 3-dimension alginate beads with IL-1 $\beta$  10 ng/ml were completed. IL-1 $\beta$ -stimulated cells induced 21.6 $\pm$ 6.3% cell death, to a significant extent, exposure of IL-1 $\beta$ -stimulated cells to contrast media induced a 31.3 $\pm$ 6.6%, indicating approximately a 10% increase in the cell death. This finding indicates that a high cell death rate of the IL-1 $\beta$ -stimulated cells is seemingly attributable to the fact that cells in inflammatory joints declined in their resistance to outside stimulation. In a normal joint cartilage, chondrocytes were surrounded by plenty of extracellular matrix and contrast medium injected into the joint was absorbed through the synovia within 3

**Fig 3.** Real time PCR was performed to measure expression of collagen type I, collagen type II and aggrecan mRNAs according to factor iohexol stimulation. The mean and standard deviation are shown. All mRNAs were not affected by 50% iohexol exposure.



hours. However the arthrography with contrast medium is applied for cases of the abnormal joint such as arthritis or cartilage damages. In such cases, cells are expected to be affected easily by the contrast medium. Cell toxicity of the contrast media could supposedly be reduced through the process of removing and washing off contrast media in the joint as early as possible after arthrography has been performed.

There are limitations in this study about the cytotoxicity of iohexol on chondrocytes. All in vitro data was obtained with cells of only one species. Moreover, it is rare to apply arthrography to bovine joint diseases. The differentiation potential of chondrocytes varies between animal species (Giannoni et al., 2005). Additional in vitro and in vivo studies are needed to determine the cytotoxicity of contrast media more clearly.

## CONCLUSIONS

This study evaluated the cell toxicity of iohexol as a contrast medium, widely used in veterinary science that utilizes bovine chondrocytes and its effects on cell metabolism. This study confirmed a significant decline in the cell proliferation at 50% iohexol. Moreover cells stimulated with IL-1 $\beta$  showed higher percentage of dead cells at 50% iohexol exposure.

In a normal joint cartilage, the cells were surrounded by plenty of extracellular matrix and the contrast medium injected into the joint was absorbed through the synovia within 3 hours of injection. However, the contrast medium is used in cases with the abnormal joint such as arthritis or cartilage defects. In such cases, where chondrocytes are frequently exposed to contrast media, the cells exposed to them are expected to be affected by the contrast medium. In conclusion, iohexol has a cytotoxicity on chondrocytes and the inhibitory effect possibly increased in inflammatory joint conditions. The repeated arthrography with iohexol should be performed with an awareness of cytotoxic effect, especially among patients that have suffered from cartilage damages and arthritis.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. ■

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