

Original Research Article

Do Nitric Oxide Synthases Enzyme Inhibitors Affect Chondrocyte Activity?

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Abstract

In the experimental models, it was observed that osteoarthritis-related clinical and histologic findings could be inhibited with nitric oxide synthase inhibitors. The aim of the present study was to investigate the effects of NO, MMP-9, MMP-13, TIMP-1 and TNF- α on the viability and proliferation of chondrocytes. Primary chondrocyte cultures were obtained from the tissues of patients undergoing osteoarthritis surgery. Cell surface antigens were examined via flow cytometric analyses. Chondrocyte cultures were evaluated for cell viability and proliferation before and after the administration of 7-NI, AG, and L-ARG were added, either alone or with TGF- β 1. Cell surface morphologies were examined via inverted light and environmental scanning electron microscopy. In all groups, TNF- α , MMP-9, MMP-13, TIMP-1 and NO were measured spectrophotometrically using commercial kits. Obtained data were analyzed statistically and $p < 0.05$ was considered significant. Locally administered neuronal nitric oxide synthase inhibitors as 7-nitroindazole were shown to selectively inhibit nitric oxide release, and increase chondrocyte proliferation significantly more than the group to which inducible nitric oxide synthase inhibitors were applied. Injecting the aforementioned medications into the knee reduces the side-effects of nitric oxide-related biochemical mechanisms to the minimum, suggesting that these medication molecules may be effective in repairing cartilage damage or decreasing cartilage degeneration?

Keywords: Aminoguanidine, L-arginine, Nitric oxide synthase, 7-nitroindazole, osteoarthritic chondrocyte, primary cell culture

INTRODUCTION

Microfracture, mosaicplasty, placement of ex vivo or in vivo scaffolds in the defect zone, and stem cell applications have been used as treatment modalities in osteoarthritis-related diseases, but the eventual results of these treatments have not been satisfactory (Niemeyer et al. 2015; Sgaglione et al. 2002). Consequently, previous studies have focused on gene therapies or tissue engineering rather than conventional treatment methods performed in clinics (Akyuva et al., 2017; Gökçe et al., 2014; Niebler et al., 2015). Researchers have recently become concerned in the use of chondrocyte cultures for biological repair (Gumustas et al., 2016; Gumustas et al. 2017; Gökçe et al. 2014; Guzelant et al., 2017; Isyar et al., 2015; Isyar et al., 2016; Yilmaz et al., 2016).

Previous studies reported that tumor necrosis factor alpha (TNF- α) and matrix metalloproteinases (MMPs) play a key role in osteoarthritis progression. Cytokines, especially TNF- α , regulate nitric oxide synthases (NOS), which are a family of enzymes catalyzing the production of nitric oxide (NO) and related inducible NO (iNOS), by signaling pathways (Kapoor et al. 2011; Miyasaka and Hirata 1997; van Buul et al. 2012). NO is essential for tissues in low concentrations and an important cellular signaling molecule involved in many physiological processes; whereas, increased levels of NO as a free radical have detrimental effects on the cells including chondrocytes (Carlo and Loeser, 2002; Mazetti et al., 2001; Min et al., 2001).

In the experimental models where NOS inhibitors were used, it was observed that osteoarthritis-related clinical and histologic findings could be inhibited (Kapoor et al., 2011; Miyasaka and Hirata 1997; van Buul et al., 2012). TNF- α also induces MMP activation which can

deteriorate the vital communication signals between the tissue inhibitors of metalloproteinases (TIMP) and the extracellular matrix (ECM), leading to chondrocyte deaths (de Mello et al. 1997; Dozin et al., 2005; Miyasaka et al., 1997; Murrell et al., 1996). With the activation of MMPs, especially type II collagens, proteoglycan synthesis is inhibited, and interleukin-1 release decreases, resulting in reduced chondrocyte proliferation, and thus fewer cell deaths (Amin et al., 1999; Goldring, 2000; Hedbom and Hauselmann 2002; Pelletier et al., 2001).

To the best of our knowledge, despite extensive research on the field, impacts of NO on the degenerative progression of osteoarthritis have not been clearly presented.

The aim of the present study was to investigate the effects of NO, MMP-9, MMP-13, TIMP-1, and TNF- α on the viability and proliferation of chondrocytes considered to be involved in the degeneration of cartilage in osteoarthritis. We used 7-nitroindazole (7-NI), aminoguanidine (AG), and L-arginine (L-ARG), added alone or with transforming growth factor beta 1 (TGF- β 1) to inhibit or activate NO production in vitro using primary chondrocyte cell cultures. Our data answers the question, "Which pathways should be inhibited or activated to decelerate the development of osteoarthritis?"

METHODS

Ethical Aspects

The study was carried out following the approval of Local

Ethics Board. Written informed consent forms from the patients were obtained.

Materials

Collagenase type II enzyme (1 mg/mL; Invitrogen Corporation), Hank's balanced salt solution (Cat#14025, Gibco), penicillin-streptomycin, fetal bovine serum, and Dulbecco's Modified Eagle's Medium (1000 mg glucose/L) were obtained from Sigma Chemical, St. Louis, USA. Sodium dodecyl sulfate (SDS) (10% L4522), Insulin-Transferrin-Selenium premix, and RPMI-1640 were obtained from Sigma-Aldrich GmbH, Germany. TGF- β 1 (Cat#H854 5 UG), 7-NI (Cat#N-7778-SG), AG (Cat#1937-19-5), and L-ARG (Cat#74-79-3) were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

The (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) (MTT) kit was Vybrant MTT cell proliferation assay (Cat#V-13154) manufactured by Cell Biolabs Inc., USA. The TNF- α (Cat#KRC3011) kit was obtained from Invitrogen. Of the enzyme-linked immunosorbent assay (ELISA) commercial kits, the NO kit was obtained from Cayman Chemical, and TIMP-1, MMP-9 and MMP-13 were obtained from eBioscience.

The laminar flow cabinet (AirFlow-NUVE; Cat#NF-800 R) and the incubator (NUVE; Cat# 06750) were obtained from Ankara, Turkey. An Olympus CKX41 was used as the invert microscope and the images obtained were recorded using Olympus cellSens software. For the analysis of viability, cell toxicity, and proliferation with commercial kits, a Mindray MR 96 A (PRC) ELISA device was used. The cells were analyzed on a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Data analysis was performed with CellQuest software (Becton-Dickinson). A Quanta 250 Field Emission Gun (FEG) environmental scanning electron microscopy (ESEM) (FeiCompany, Hillsboro, Oregon, USA) was used for cell surface morphology.

Patient selection and study design

Tissues used for primary chondrocyte cultures were obtained from patients (n=6) who had undergone surgery for osteoarthritis. Primary chondrocyte cultures (considered as passage number:0) reached at 85% confluency, and were detached from culture vessel using trypsin and placed into new culture flasks. Acquired monolayer chondrocyte cultures were used in subsequent experiments. Cell surface antigens were examined via immunoflow cytometric analyses.

Chondrocyte cultures obtained from each patient were evaluated for cell viability, toxicity, and proliferation before and after the administration of 7-NI, AG, and L-

ARG were added, either alone or with TGF- β 1. Cell viability, toxicity, and proliferation tests were administered at 0hour, and on the days 7 and 15.

Cell surface morphologies of the control and experimental groups were examined via invert light microscopy, and ESEM. In all groups, TNF- α , MMP-9, MMP-13, TIMP-1, and NO were measured spectrophotometrically using commercial kits.

All analyses regarding primary chondrocyte cultures were carried out by the same researchers to keep the errors at a minimum level. The researcher conducting the molecular analysis was blinded to the agent added to the well, the experimental design, and the chemicals administered. All experiments were carried out three times.

Patients who had used medications possibly affecting TNF- α (such as DMARDs and/or etanercept, infliximab, adalimumab, abatacept, and rituximab) in the previous two years, or the patients who had used nitroglycerin-containing medications (which may affect the NO levels during evaluations), and/or vasodilators (which may release the amyl-nitrite group) were excluded from the study. Tissues were obtained from all patients included in the study (n=6).

Preparation of the medications and primary chondrocyte cultures

Powdered forms of the following agents were dissolved in proper solvents per manufacturer manuals: 7-NI (selectively inhibiting neuronal NOS [nNOS]), AG (iNOS), LARG (increasing NO synthesis) (Yilmaz and Ulugol 2009) and the growth factor TGF- β 1 (stimulating the chondrocyte proliferation) under aseptic conditions (Gumustas et al. 2017). These solutions were then aliquoted as: L-ARG 3 μ L/mL (Badger et al. 1998), 7-NI 200 μ L/ml (Cheng et al. 2014), AG 1 μ L (Otero et al. 2005), and TGF- β 1 5 μ L/ml (Ratnayake et al., 2014), (each per mL) and kept at 4°C prior to use.

They were transferred to dark brown, tightly sealed bottles and letter coded to blind the researchers to the agents used. Chemical agents, commercial stock solution concentrations, applied volumes and final concentrations are given in (Table 1).

The study included the tissues that remained after the exclusion criteria were applied. These were obtained from patients (n=6) with advanced stage osteoarthritis who had not responded to medical or conservative treatments and underwent total knee arthroplasty (mean age 66 years; Stage 4 gonarthrosis according to Kellgren-Lawrence stages (Kellgren and Lawrence 1957) (Figure 1).

Tissues from lateral and medial femoral condyles, as well as the tibial plateau, are routinely removed during total knee arthroplasty and treated as surgical waste.

Table 1. Agents; commercial stock solution concentrations, applied volumes and final concentrations.

Pharmacological Agents	Commercial Stock Solution Concentration (g/100 mL)	Applied Volumes ($\mu\text{L/ml}$)	Final Concentration ($\mu\text{L/mL}$) *	Solvent
L-ARG	200	3	9	UPW
7-NI	50	200	600	UPW
AMG	30	1	3	UPW
TGF- β 1	0.001	5	15	BSAHM

AMG: Aminoguanidine; L-ARG: L-arginine; 7-NI: 7-nitroindazole; TGF- β 1: transforming growth factor beta 1, UPW; Ultra-pure water, BSAHM; Bovine Serum Albumin and HCl mixture (:1 well is a 3-mL volume).

**Figure 1.** Radiological image of a stage IV patient.

These osteochondral tissues were preserved during the surgeries of the study patients and transferred to cell culture laboratory under sterile conditions. Standard primary culture protocols were performed to obtain a monolayer human primary chondrocyte culture (Isyar et al., 2016; Yasar Sirin et al., 2017).

Chondrocytes were detached from the culture vessel using trypsin-ethylenediaminetetraacetic acid, and then cells were stained with Trypan blue and counted using Thoma lams. In addition, immunoflow cytometric analyses were performed on these primary chondrocytes. A culture sample of 125×10^3 cells was placed in each of 24-well plates and kept in incubator for 48 hours. Cell culture media were refreshed every other day. Chemical agent application was performed when chondrocyte cultures reached 85% confluency.

Primary chondrocyte cultures comprised the control group before the addition of chemical agents. The agents were administered to primary cultures using micropipettes twice a day at the same time in the morning and evening. Within the boundaries of the total posology of the study design, each agent was added to each cell culture sample alone or in combination (Table 2).

Analyses

Cell morphology and confluency were analyzed using an inverted microscope. Micro-photographs of the cell organizations were obtained in the confocal/contrast phase before and after chemical agent applications, at 4X, 10X, 20X and 40X magnifications.

Table 2. Pharmacological agents applied according to the groups.

Groups	Added agents
Group I (n=6)	CCM
Group II (n=6)	TGF- β 1 + 7-NI
Group III (n=6)	TGF- β 1 + AG
Group IV (n=6)	TGF- β 1 + L-ARG
Group V (n=6)	CCM+ 7-NI
Group VI (n=6)	CCM+ AG
Group VII (n=6)	CCM+ L-ARG
Group VIII (n=6)	CCM+ TGF- β 1

AMG: Aminoguanidine; L-ARG: L-Arginine; 7-NI: 7-Nitroindazole; TGF- β 1: Transforming growth factor beta 1, and CCM: Cell Culture Medium.

Cell surface expression levels were obtained before the administration of the chemical agents via a flow-cytometer. For this purpose, chondrocytes were scratched off the surface following the application of EDTA-trypsin (0.25%), and transferred into tubes and centrifuged twice consecutively at 2000 rpm and 4°C for 5 minutes. Precipitated pellets were re-suspended using the cell culture medium. Cells (about 0.5×10^5 per ml) were incubated protected from light at 22.4°C for 20 minutes with surface markers fluorescein isothiocyanate (FITC) and phycoerythrin (PE), conjugated monoclonal antibodies (HLA-DR, CD10, CD11b, CD14, CD34, CD45, and CD117), and proper isotype controls. Then, the medium content was washed by centrifuging at 1,300 rpm and 4°C with the addition of phosphate buffer saline pH=7.4 with 0.1% sodium azide. Resuspended cells were examined with flow cytometry and analyzed with software, and the images were reported (Isyar et al., 2016; Yasar Sirin et al. 2017).

ESEM analysis was carried out to assess the surface topography and composition of the samples. A device with a lifting system and having the ability to transfer the electron beam in a high vacuum was used. This enabled us to obtain images of the extracellular matrix, as well as the characteristic cellular structures. FEG ion pumps were used for the high vacuum. Images were recorded at a pressure of 100-220 Pa in ESEM vacuum mode, under magnifications of 1,000-160,000x, at resolution depths (HFW) of 41.4 and 414 μ m, at an operating voltage of 5.00 kV, and at a wavelength dispersive (WD) of 8.8-8.9 mm.

ELISA analyses were performed at 0 hours and on days 7 and 15 using microplate readers in line with the commercial kits' manuals. Viability tests were carried out by commercial MTT kit parallel to the manufacturer's instructions. NAD(P)H-dependent mitochondrial oxidoreductase enzymes reduce the tetrazolium dye MTT to its insoluble purple formazan crystals in live cells (Isyar et al., 2016; Yasar Sirin et al., 2017).

A 12 mM MTT stock solution was prepared by adding 1 mL of sterile phosphate buffer saline to one 5 mg vial of

MTT. Next, 10 mL of 0.01 M HCl was added to one tube containing 1 g of SDS and mixed until the SDS dissolved. After removing cell culture media, 1 ml of fresh culture medium and 100- μ L of MTT stock solution were added per well. These were incubated at 37°C for 2 hours, protected from light. Afterward, 500 μ L of DMSO was added to each well and mixed thoroughly with a pipette, then incubated at 37°C for an additional 10 minutes prior to photometric analysis of its 540-nm-wavelength absorbance. For the assessment of proliferation, 500- μ L of SDS-HCl solution was added to the remaining cells and incubated at 37°C for 18 hours.

The reaction solution was subjected to photometric analysis at 570-nm wavelength. The vitality of the control group was assumed to be 100% prior to addition of agents (at 0 hours) to the culture medium. Cell viability was recorded at 0 hours and on days 7 and 15 after the administration of the agents, and measurements of proliferation were recorded. In this manner, intergroup comparisons in terms of viability, proliferation, and toxicity after the application of chemical agents (7-NI, AG, and L-ARG) were performed.

MMP-9, MMP-13, and TIMP-1 were directly read at the 450-nm absorbance band, and TNF- α was directly read at the 490-nm absorbance band, using the ELISA microplates with commercial kits and according to their instructions. NO was evaluated at the 540-nm wavelength via nitrate and nitrite values.

SPSS Statistics 18.0 was used for statistical analyses. Data were expressed as mean \pm standard deviation. Analysis of variance (Repeated measures ANOVA) was used for data analyses in repeated measures. Levene's test was used to assess homogeneity of variances. Mauchly's Test of Sphericity was performed to analyze the significance of differences in/between variances. Bonferroni test was used to assess a paired comparison (post-hoc test) for variances with significant difference as a result of ANOVA. $P < 0.05$ was adopted as the probability value.

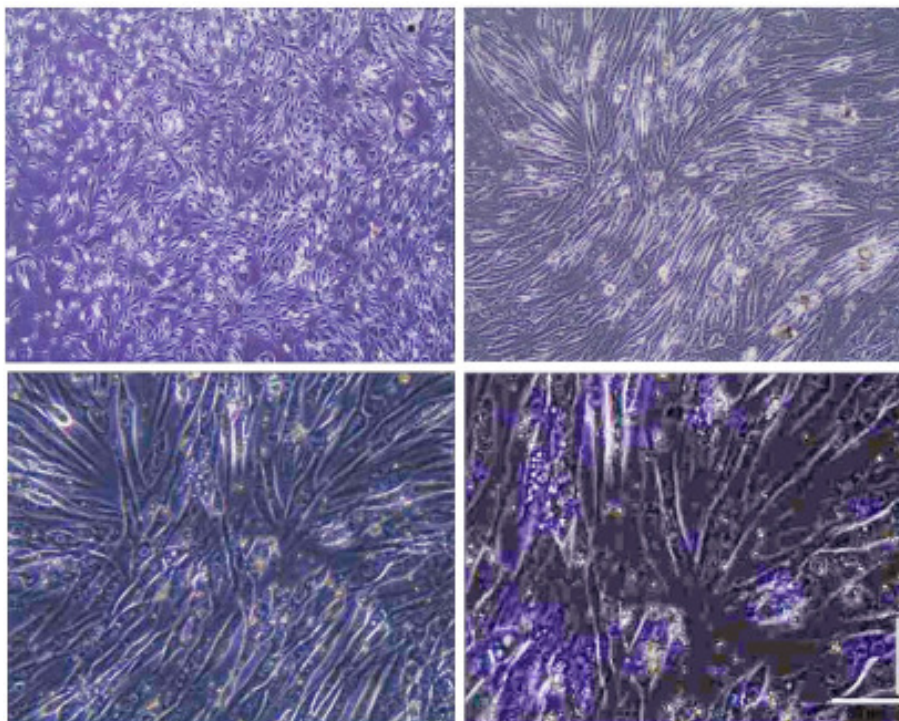


Figure 2. Microimages obtained via inverted light microscopy.

RESULTS

Invert microscopy evaluations

The cells were confirmed to be adhered to the adhesive surfaces of the flasks and wells. They were healthy, and they proliferated (Figure 2).

Immunoflow cytometric evaluations

Chondrocytes did not express CD34, CD14, CD45 (non-significant) surface markers, but did express CD71, CD73, and CD105, all of which are typical markers for cells.

The expression results of these immunophenotypical markers indicate that the primary chondrocyte cultures were healthy.

Evaluations of the ESEM microphotos

On day 15, in the experimental group in which enzyme blockage was attained neuronally by 7-NI, chondrocyte proliferation was healthier compared to the control group, which was indicated by isogenous, circular, and bright images of the nesting chondrocytes and their extensions (Figure 3).

Unlike Group V (7-NI group), the images of the samples treated with TGF + 7-NI, TGF + L-ARG, or TGF + AG showed altered cell morphology and extracellular matrix composition.

Statistical evaluation of MTT-ELISA results

NO results

Descriptive statistical results concerning all variances of eight working groups were given in Table 1. A significant difference was found among them ($p < 0.05$). Post-hoc Bonferroni test was performed to determine the source of the differences. As a result of the test, it was concluded that all groups had differences ($p < 0.05$).

It was found that there were significant differences after measurements (0th hour, 7th day and 15th day) ($p < 0.05$). Post-hoc Bonferroni results indicated significant differences for all three measurements. ($p < 0.05$).

MMP-9 results

A significant difference was found among the 8 working groups ($p < 0.05$). Post-hoc Bonferroni test was performed to determine the source of the differences. As a result of

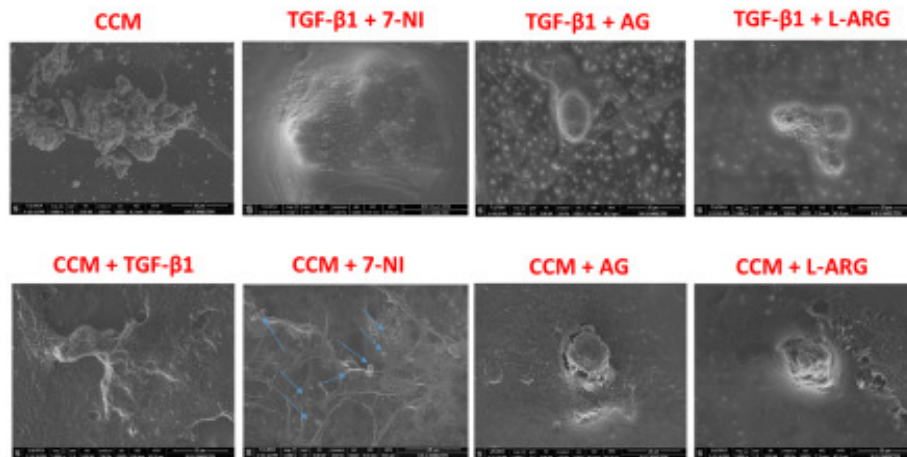


Figure 3. ESEM images supporting the MTT-ELISA viability, toxicity, and proliferation as well as inverted light microscopy.

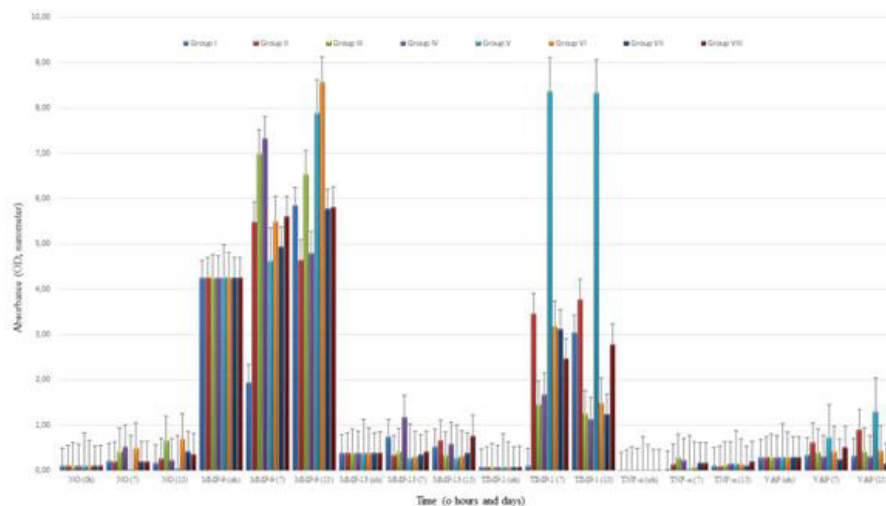


Figure 4. Statistical results of MTT-ELISA cell viability, toxicity, and proliferation.

the test, it was concluded that all groups had differences ($p < 0.05$).

As a consequence of the measurements, there were significant differences (0th hour, 7th day and 15th day) ($p < 0.05$).

Post-hoc Bonferroni test results indicated significant differences for all three measurements ($p < 0.05$).

MMP-13 results

A significant difference was found among the 8 working groups ($p < 0.05$). Post-hoc Bonferroni test was performed to determine the source of the differences. As a result of the test there was no significant difference between the 3rd and 8th groups ($p > 0.05$). All other groups had a significant difference ($p < 0.05$).

As a consequence of the measurements there were significant differences (0th hour, 7th day and 15th day) ($p < 0.05$). Results of Post-hoc Bonferroni test were significant for 3 measurements ($p < 0.05$).

TIMP-1 results

A significant difference was found among the 8 working groups ($p < 0.05$). Post-hoc Bonferroni test was performed to determine the source of the differences. As a result of the test, it was concluded that all groups had differences ($p < 0.05$).

As a consequence of the measurements, there were significant differences (0th hour, 7th day and 15th day) ($p < 0.05$). Post-hoc Bonferroni results indicated significant differences for all three measurements ($p < 0.05$).

TNF- α results

A significant difference was found among the 8 working groups ($p < 0.05$). Post-hoc Bonferroni test was performed to determine the source of the differences. As a result of the test, the results were as follows:

- A difference was found between the 1st and the 3rd groups ($p < 0.05$).
- A difference was found between the 1st and the 4th groups ($p < 0.05$).
- A difference was found between the 3rd and 5th groups ($p < 0.05$).
- There was no difference among other groups ($p > 0.05$).

There were significant differences as a result of the measurements (0th hour, 7th day and 15th day) ($p < 0.05$). Post-hoc Bonferroni test was performed to determine the source of the differences. As a result of the test, the results were as follows:

- A difference was found between the 1st and the 2nd groups ($p < 0.05$).
- A difference was found between the 1st and the 3rd groups ($p < 0.05$).
- There was no difference between the 2nd and the 3rd groups ($p > 0.05$).

MTT-ELISA cell viability, toxicity, and proliferation (V&P) results

A significant difference was found among the 8 working groups ($p < 0.05$). Post-hoc Bonferroni test was performed to determine the source of the differences. As a result of the test, the results were as follows:

- There was no difference between the 1st and the 4th groups ($p > 0.05$).
- There was no difference between 3rd and 6th groups ($p > 0.05$).
- There were differences in all other groups ($p < 0.05$).
- As a result of the measurements, it was found that there were significant differences (0th hour, 7th day and 15th day) ($p < 0.05$). Results of post-hoc Bonferroni test indicated that there were significant differences in 3 measurements ($p < 0.05$).

DISCUSSION

Current orthopedic studies mostly focus on pharmaceutical and molecular approaches aiming to delay and/or ameliorate the cartilage damage in joints (Bulman et al., 2015; Dogan et al., 2016; Yasar Sirin et al., 2017).

Such studies are usually conducted on cell lines, or on cultures obtained from animal tissues. As is known, animal models are necessary but quite different from human tissues, especially in the sensitivity they show to medicine, which may yield different and misleading

results (Gumustas et al., 2016; Gumustas et al., 2017; Isyar et al., 2016).

On the other hand, commercial cell lines are widely used in orthopedic research, but there are some limitations of studies based upon them. The main disadvantages of these commercial cell lines are that they involve only one type of cell and do not possess complex coordination mechanisms in relation to their microenvironments.

Eventually, since the interaction of these cells with elements such as ECM are inhibited, already problematic in vitro test results become more problematic, due to their in compliance with in vivo conditions. Another disadvantage of these cell lines is that they do not carry human genotypical and phenotypical features, since most of them are genetically modified (Gumustas et al., 2016; Gumustas et al., 2017; Isyar et al., 2016).

In the present study, all chemical agent applications were performed on primary chondrocyte cultures in vitro. Results obtained from primary cell cultures are much more reliable, compared to cell-lines and animal-tissue studies, because a primary culture represents all cell types and ECM components of human tissue. The only limitation of our present study is that no comparison was made between the cartilage obtained from the patient and healthy cartilage tissues, given that it is not ethically possible to obtain healthy cartilage tissue from a healthy person.

Existing studies of tissue cartilage protection or repair mainly focus on cartilage cell apoptosis attained by cytokines, such as TNF- α , and by various modulators, such as MMP. It has been reported that cartilage destruction occurring in osteoarthritis is a possible result of catabolic factors released by chondrocytes (Guzelant et al., 2017; Dozin et al., 2005).

The catabolic pathway of joint cartilage has been explained as follows: Collagens and proteoglycans of the ECM are destroyed by the MMPs released by chondrocytes; the degraded matrix molecules increase cytokines, like TNF- α , and NO expression is stimulated (Dozin et al., 2005; Fosang et al., 2008; Poole et al., 2002). Additionally, the anabolic pathway has been explained as: Due to the increase in proteoglycans and chondrocyte proliferation via TGF- β 1, TIMPs are stimulated, and thus the harmful effects of cytokines are inhibited (Dozin et al., 2005; Fosang et al., 2008; Goldring and Goldring, 2004). Therefore, researchers have tried to inhibit NO, which causes an oxidative destruction in tissue and joint cartilage, by activating ECM destruction. Consequently, the clinical use of NOS inhibitors has produced reports of their protective effects on experimental arthritis models and joints (Parker et al., 2013; Rosa et al., 2008).

It has been reported in the literature that the proteinases found in osteoarthritis, causing ECM destruction, were MMP-13 and MMP-9, both of which

destroy collagen (Birkedal-Hansen, 1993); however, another study reported the protective effect of MMP-13 on the destruction of cartilage with osteoarthritis (Levin et al., 2001; Yilmaz et al., 2013). Swan et al. reported that the increase in TIMP-1 was in favor of cartilage protection (Swan et al., 1974). Clegg et al. reported that TIMPs, which are the local inhibitors of metalloproteinase, strongly inhibited MMP activities (Clegg et al. 1998).

In our study, to stimulate the anabolic and catabolic reaction of NO in human osteoarthritic cultures, we have used 7-NI, AG, and L-ARG. In addition, to maintain chondrocyte proliferation and to increase proteoglycan production, TGF- β 1 was added to some of the experimental groups. We then investigated the viability and proliferation of the chondrocytes in the presence of TNF- α , MMP-9, MMP-13, and TIMP-1, which are indicated as mediators of cartilage catabolism.

In this study, a strong positive correlation between NO and MMP-9/TNF- α was observed; however, the correlation of NO with MMP-13 and MMP-9 was negative ($p < 0.05$).

There was a strong positive correlation between TIMP-1 and NO ($p < 0.05$). There was an increase also in chondrocyte proliferation in Group V, where TIMP-1 increased ($p < 0.05$).

Another experimental study noted that arthrosis development could be decreased by the selective inhibition of iNOS; however, the study did not present clear effects of the selective inhibition of nNOS on cartilage damage mechanisms (Gokay et al., 2016; Qi and Scully, 1997).

In the present study, healthy chondrocyte proliferation was allowed in the nNOS group rather than the iNOS group from day 7 to day 15. In the group to which 7-NI was administered, a decrease was observed in NO, TNF- α , MMP-9, and MMP-13 levels, whereas an increase was observed in the TIMP-1 level ($p < 0.05$).

Intergroup comparisons on days 7 and 15 showed that, in the 7-NI group where healthy chondrocyte proliferation occurred, the decrease in nitric oxide, TNF- α , MMP-9, and MMP-13 levels, as well as the increase in the TIMP-1 level, were statistically significant ($p < 0.05$). Other than the Tukey analysis following the variance analysis, a Pearson correlation test showed a positive strong correlation of NO with MMP-9 and TNF- α . In addition, the correlation of NO with MMP-13 and MMP-9 was observed to be weak and negative. The more important finding was that a strong positive correlation was observed between TIMP-1 and NO. The correlation of cell viability and proliferation with NO and MMP-13 was found to be weak and negative. However, we observed a strong positive correlation of chondrocyte viability and proliferation with TIMP-1.

Along with iNOS inhibitors, which have been widely discussed in the literature, locally administered nNOS

inhibitors (Group V in which 7-NI was used) were also shown to selectively inhibit NO release, and they increased the chondrocyte proliferation significantly more than the group in which iNOS inhibitors were used ($p < 0.05$).

As a result, for the oxidative stress-related damages to the cartilage tissue, the local use of 7-NI, an nNOS inhibitor, as a treatment modality must be investigated *in vivo*.

CONCLUSION

Injecting the aforementioned medications into the knee reduces the side-effects of nitric oxide-related biochemical mechanisms to the minimum, suggesting that these medication molecules are effective in repairing cartilage damage and decreases cartilage degeneration. After successful completion of therapeutic studies, 7-nitroindazole may be worth considering for incorporation into orthopedic medicines.

Conflict of Interest

The authors declare no conflicts of interest.

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