EXPERIMENTAL STUDY

Acta Orthop Traumatol Turc 2014;48(3):313-319
doi: 10.3944/AOTT.2014.2635

Does insulin, transferrin and selenous acid preparation effect chondrocyte proliferation?

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Objective: The aim of this study was to test the hypothesis that insulin, human transferrin, and selenous acid (ITS) preparation have positive effects on chondrocyte proliferation and morphology and investigate the biochemical and histological effects of these additive substances in different cell culture media.

Methods: Human cartilage-derived cells (hCDCs) were isolated from the cartilage tissue of a 57-year-old woman diagnosed with gonarthrosis. Tissue samples were cultured in Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640. The cells' chondrogenic activities were observed. After serial passagings, cells were divided into 4 groups at the end of the 6th week. On the 14th day, proliferated cells were examined using an inverted microscope with x4, x10, x20 and x40 magnification and microphotographs were taken. Living cell quantity was determined on the first and 14th days using MTS-ELISA cell proliferation assay.

Results: DMEM (without adding ITS premix solution) and RPMI-1640 containing ITS premix solution provide proliferation of the chondrogenic cells. The proliferation and viability of chondrocytes were revealed in this study in the 3rd group (DMEM solution without additives).

Conclusion: It is suggested that the culture medium ingredients play crucial roles on chondrogenic proliferation in osteochondral tissue cultures.

Key words: Cell culture; chondrogenic differentiation; DMEM; ITS premix solution; RPMI-1640.

Cell behavior may vary in different culture environments depending on their features.[1,2] It is known that the media content of cell cultures (MCCC) and their amounts are important to direct the differentiation of the primary cell cultures and stem cells into the desired cell type.[1,2] While MCCC provide the micro-environment necessary to maintain normal metabolic activity of cells in laboratory condition, they differ according to capable of adaptation, cell source and organism type.[1,2]

Additives, such as amino acids, carbohydrates, lipids, vitamins and ions added to the MCCC for the vitality and proliferation of the cells used *in vitro* cell culture experiments also preserve and support their further development.[1-4]

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Submitted: February 09, 2011 **Accepted:** February 01, 2012

Available online at
www.aott.org.tr
doi: 10.3944/AOTT.2014.2635
QR (Quick Response) Code

Studies on the addition of various additives into the culture environments used in the preparation of the MCCC have been reported.[5-16] The results of these studies have shown that the presence of additives to the MCCC is very important for the proliferation of cells in addition to proper pH, temperature and humidity *in vitro*. The current literature does not contain any study on the quantification of the proliferative effect of the MCCC.[17]

In this study, we aimed to investigate the culture medium and the additive ingredients necessary to manage the chondrocytic differentiation of the primary cell cultures and obtain the optimal cell culture when preparing the medium content.

Materials and methods

Two commonly used commercial MCCC products were used in the current study; DMEM (Dulbecco's modified Eagle's medium (1000 mg glucose/L); Sigma-Aldrich Corp., St. Louis, MO, USA) and RPMI-1640 (Mega-cell RPMI Medium 1640 (x1) liquid; Sigma-Aldrich Chemie GmbH, Munich, Germany). ITS premix solution (insulin, human transferrin, selenic acid powder (I1884); Sigma-Aldrich Corp., St. Louis, MO, USA) was added as an enrichment additive. Proliferation and differentiation results of chondrocytes were examined.

A 57-year-old female patient with symptomatic gonarthrosis which was unresponsive to other treatments underwent total knee arthroplasty. After obtaining a signed informed consent form, osteochondral tissue residue harvested from the femoral lower condyle was placed in RPMI-1640 and DMEM.

Osteochondral tissue was treated with lyophilized Type 2 collagenase enzyme (1 mg/mL; Invitrogen Corp., Carlsbad, CA, USA) at 37.4°C for 24 hours. Cells were then centrifuged at 4°C. The supernatant was discarded. Penicillin-streptomycin (Penicillin-Streptomycin Solution; Sigma-Aldrich Chemie GmbH, Munich, Germany) and fetal bovine serum (Fetal Bovine Serum; Sigma-Aldrich Corp., St. Louis, MO, USA) were added to the cream-colored cell pellet formed at the bottom of the falcon tube. RPMI-1640 or DMEM were added MCCC with additives according to the study groups and cells were transferred into T25 flasks (Tissue culture flask product no. 90151; TPP Techno Plastic Products AG, Trasadingen, Switzerland) with a surface area of 25 cm^2. Human cartilage cells were cultured under standard cell culture conditions. Cell cultures reached 80-85% confluence on the 6th passaging at 6 weeks. Cells were then transferred into 60 mm^2 culture petri dishes in the amount of 1 ml MCCC per 4 ml cell suspension.

Cell cultures were collected from flasks with trypsinization after the confluent reached the desired amount. Living/dead cell ratio was detected through the Thoma chamber using a 0.4% Trypan Blue solution. Living cells, with a number of $4.7x10^5$, were seeded into petri dishes.

Test samples were divided into four groups according to their MCCC. RPMI-1640 was added to Group 1, ITS premix solution with RPMI-1640 was added to Group 2, DMEM to Group 3 and ITS premix solution with DMEM in Group 4 (Table 1).

Extra cell groups were reserved for each study and the control groups for the MTS viability and toxicity analysis (1st and 14th days).

The supernatants of primary cell culture mediums of each group were changed with their corresponding MCCC every two days.

Petri dishes containing primary cell cultures were

Table 1. The media content of cell cultures (MCCC) according to groups.

	Group 1	Group 2	Group 3	Group 4
RPMI-1640 (x1) (88% in total amount of MCCC)	+	+	–	–
DMEM (x1) (1,000 mg glucose/L) (88% in total amount of MCCC)	–	–	+	+
ITS premix solution [Insulin (5 μg), human transferrin (5 μg), selenic acid (5ng/ml)] (1% in total amount of MCCC)	–	+	–	+
Fetal bovine serum (12% in total amount of MCCC)	+	+	+	+
Penicillin-Streptomycin (1% in total amount of MCCC)	+	+	+	+

suspended in an incubator (Thermo Scientific, 321-315684/467; Thermo Fisher Scientific Inc., Rockford, IL, USA) with 5% CO_2 at 37.4°C for 14 days. MCCC was changed in a volume of 5 ml every two days based on the content of each group.

Five ml of ITS premix solution (insulin 5 µg, human transferrin 5 µg and sodium selenite 5 ng/ml mixture) was added to MCCC in Group 2 and 4, as a supplement for chondrocytic differentiation.

Microphotographic images were obtained at a magnification of x4, x10, x20 and x40 for histological evaluation on the 1st and 14th days using an invert microscope (Olympus inverted microscope CKX41; Olympus Corp., Tokyo, Japan).

Viability tests were performed using MTS formazan commercial kit (MTS Cell Titer 96®, one solution Aqueous Non-Radioactive Cell Proliferation Assay; Promega Corp., Madison, WI, USA) in accordance with the manufacturer's instructions on the 1st and 14th days.

Viability and toxicity tests were performed with both macroscopic and microscopic examinations using an MTS commercial kit (100 ml MTS solution + 5 ml of phenazine methosulfate: phenazine methosulfate solution [PMS]) on the 1st and 14th days.[18-21] For this purpose, the media solution with MTS was prepared in a dark room with a ratio of 1/6. On the 1st and 14th days, extra reserved cell groups for each group and the cell culture media within control groups were removed with a pipette gun and discarded. A 2,500 µL mixture of MTS tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymeth-oxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and culture medium solution containing DMEM, each, were added to each petri dish. The cells were left for a period of 3 hours in an incubator (5% CO2 and 37.4°C).

Test groups were then taken from the incubator. To stop the reaction, 125 µl of 10% sodium dodecyl sulfate (10% SDS L4522; Sigma-Aldirch Chemie GmbH, Munich, Germany) were added to each well. MTS solutions of 300 µl were transferred from the petri dishes to a 96-well plate and analyzed at a wave length of 490 nm using an ELISA Microplate Reader (MR-96A; Shenzhen Mindray Co. Ltd., Shenzhen, PRC) device.

Data were analyzed using SPSS 17 package software. Statistical analysis results were evaluated via cell proliferation. Variance analysis (ANOVA) test and the F-test were used for intergroup comparison.

Results

Cell proliferation ratios were calculated by dividing the absorbance results of negative controls to absorbance values for the experimental groups on the ELISA plate

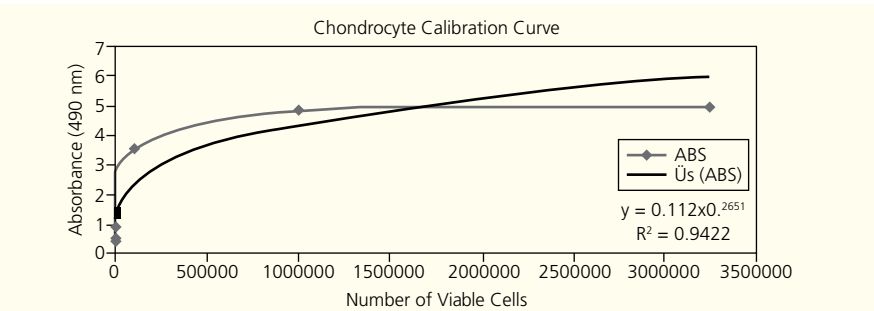

Fig. 1. Standard calibration curve expressing number of viable cells on the 1st day.

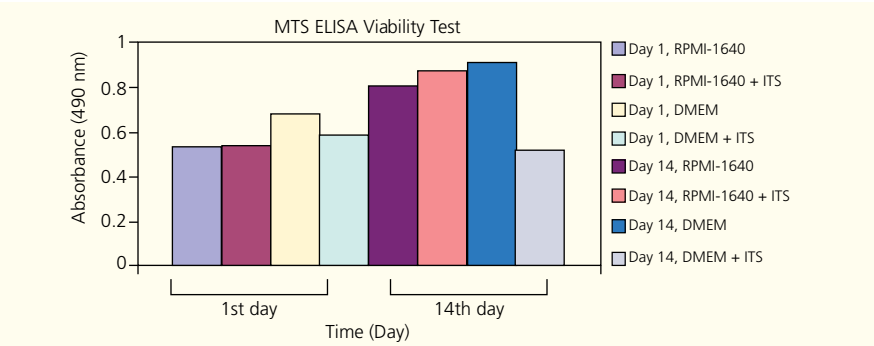

Fig. 2. Absorbance graphic of MTS ELISA viability test results on the 1st and 14th days. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

reader and are given as a percentage. Absorption results of the control group were considered as 100% cell viability. A comparative standard calibration curve, as well as absorbance results expressing the number of viable cells on the 1st and 14th days, are given in Figures 1 and 2.

No significant differences were found between Group 1 and Group 2 on Day 1. However, the number of living cells in Group 3 was higher than those in the other groups. The difference in the number of living cells between Group 1 and 2 was significant at the end of the 14th day and the number of living cells was higher in Group 2.

According to the MTS ELISA analysis, cells in Group 3 reached the highest quantity at the end of the 14th day.

The proliferation of cells by colony formation seen in inverted microscope examination at the end of Day 14 was confirmed by the MTS ELISA viability test. Maximum cell growth was observed mainly in Group 3, then in Group 2 and Group 1. The least amount of cell growth was observed in Group 4 (Figs. 3 to 6).

There was a statistically significant difference between the groups containing RPMI-1640, RPMI-1640 + ITS premix solution, DMEM and DMEM + ITS premix solution on the 1st and 14th day ($p=0.0001$ and $p=0.008$, respectively) (Table 2 and 3).

Discussion

The combination of essential amino acids in cell cultures

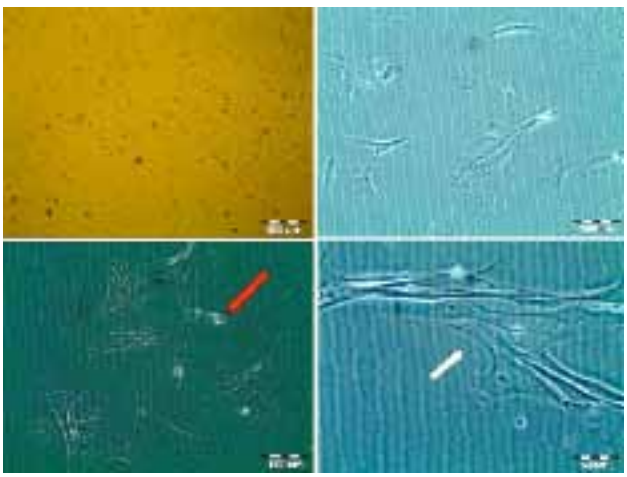

Fig. 3. Invert microscopic images (x4, x10, x20, x40) of human cartilage-derived chondrocytes with the presence of RPMI-1640 on the 14th day. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

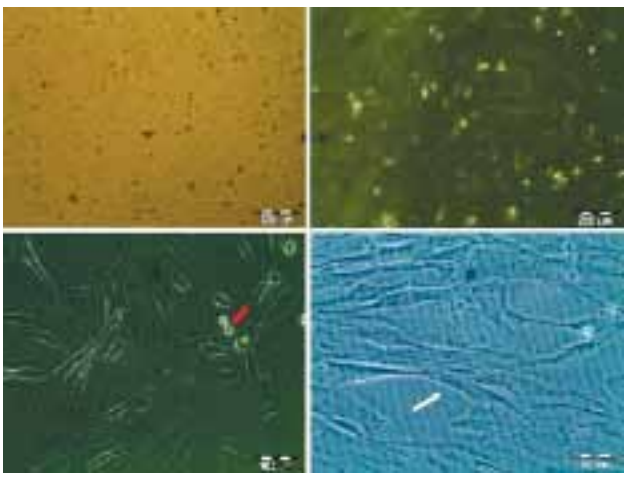

Fig. 5. Invert microscopic images (x4, x10, x20, x40) of human cartilage-derived chondrocytes with the presence of DMEM on the 14th day. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

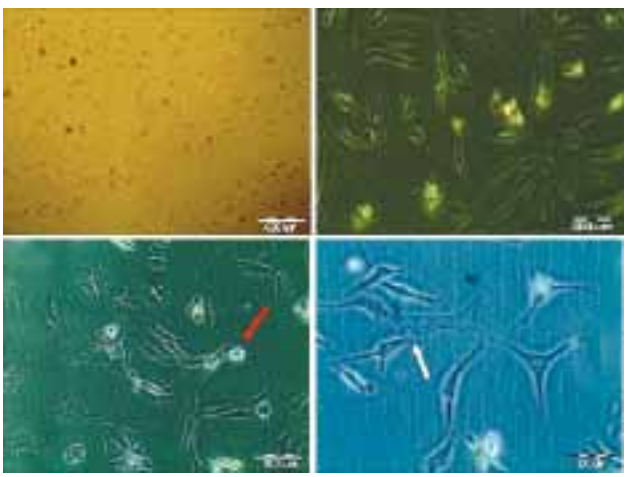

Fig. 4. Invert microscopic images (x4, x10, x20, x40) of human cartilage-derived chondrocytes with the presence of RPMI-1640 + ITS premix solution on the 14th day. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

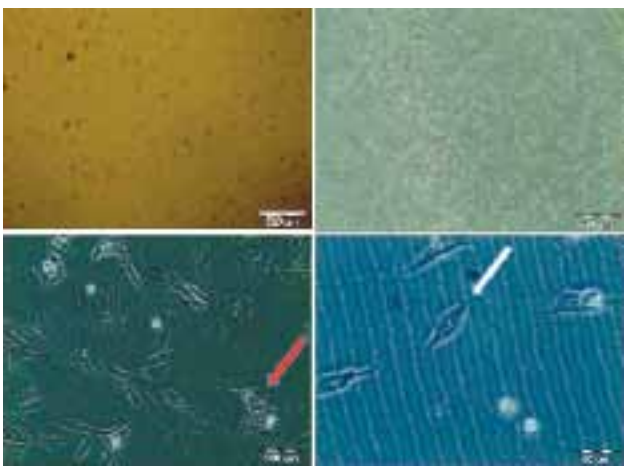

Fig. 6. Invert microscopic images (x4, x10, x20, x40) of human cartilage-derived chondrocytes with the presence of DMEM + ITS premix solution on the 14th day. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Table 2. Statistical comparison of MTS ELISA viability test results between groups on the 1st day.*

Time (Day)	Squares of sums	df	Mean square	F	p
1st day					
Intergroup	0.043	3	0.014	14338.000	0.0001
Intragroup	0.000	8	0.000		
Total	0.043	11			

*ANOVA

Table 3. Statistical comparison of MTS ELISA viability test results between groups on the 14th day.*

Time (Day)	Squares of sums	df	Mean square	F	p
14th day					
Intergroup	0.284	3	0.095	8.170	0.008
Intragroup	0.093	8	0.012		
Total	0.377	11			

*ANOVA

was first described in 1955 by Eagle.[18] The minimum Eagle's medium (MEM) is still used as a cell culture medium with some modifications. Dulbecco's modified Eagle's medium (DMEM) contains glucose, essential amino acids and vitamins for nourishing cells at desired osmolarity and pH to sustain their vitality and functions.[19] Components of the medium contain amino acids, vitamins, salts, glucose, antibiotics, and serum for the culture of mammalian cells.[20] Therefore, the needs of cell culture media must be determined according to the purpose of the study. The necessity of amino acids, carbohydrates, lipids, vitamins, ions and proteins for continuation of viability and proliferation of the cells has been previously emphasized.[1] The required contents of cell culture media may vary by the cell type, adaption capability and organism cell source type.

It has been reported that controlling the cell culture phase is essential for better chondrocytic proliferation and differentiation.[17,21]

Additionally, the use of stimulating mediators for developing intercellular network with proliferation and differentiation of cells has been reported.[22-24] The paucity of obtained number of cells and limited proliferation and differentiation capabilities are disadvantages in terms of *in vitro* and *in vivo* applications.[25,26]

There are no sufficient reported clinical long-term follow-up outcomes of mesenchymal stem cell-derived cartilage tissue transplantations. However, studies examining the characteristics of cell group responses to biological stimulations offer promising preliminary results considering the forthcoming success of tissue engineering applications.[20] In this context, the ultimate success of the cell culture media is dependent on the preferences of the MCCCs. The necessity of the use of local regulating mediators, which reduces cell death and disintegration for cell proliferation and intercellular network production, is one of the important details in treatment of chondral lesions. Therefore, optimization of the co-existence of the cells and local regulators is required. This necessitates the solution of a complex process.[20,21]

Gomez-Camarillo et al.[27] tested different cell culture systems for increasing chondrocyte cell proliferation on articular cartilage of normal and osteoarthritis male Wistar rats. They compared growth factors as MCCC and DMEM without growth factors, which consisted of phosphate salt buffer solution, glucose, sodium and secondary phosphate (weak acidic pH) as the control group in proliferation and differentiation of chondrocytes cultures. They observed a significant increase in the number of proliferated chondrocytes obtained from osteoarthritic cartilage tissue in enriched DMEM.

Ceyhan et al.[28] studied osteoblast cultures derived from bones of the skull of adult and fetal Balb/c type mice. They used DME-F12 in the fetal and four adult calvariums and RPMI-1640 medium in six adult calvariums to standardize osteoblast cell culture techniques. It was shown that embryonic tissues could be cultured with higher rates and that, similar to cartilage cell culture studies, MCCC plays an important role in the success of proliferation and differentiation of bone cell cultures.

Yu et al.[29] added ascorbic acid-2-phosphate and proline in DMEM/F-12. The cultured cells were transferred to full-thickness cartilage defect area of New Zealand rabbits and examined histopathologically after the

12th week. They emphasized the importance of the ingredients added to medium for cell proliferation.

Priddy et al.[30] examined cells morphologies and formation of extracellular matrix of cultured chondrocytes harvested from humeral head cartilage of adult dog cadavers. They used RPMI-1640 (R), ascorbate wih RPMI-1640 (RA), Ham's F-12 (F), and ascorbate with Ham's F-12 (FA) as the MCCC and analyzed the matrix formed by chondrocytes in all four groups for 20 days immunohistochemically. The quantity of produced glycosaminoglycan, and collagen Type 2 was higher in Group R and RA clusters of chondrocytes than chondrocyte clusters of F and FA groups from the 10th day up to the 20th day.

Zhang et al.,[31] on the other hand, using mesenchymal stem cells in goat's bone marrow in their *in vitro* test model, observed chondrogenic differentiation. The chondrogenic differentiation was investigated through the addition of insulin, transferrin, vitamin C and DXM into DMEM containing high-glucose as the MCCC. They observed an increase of the chondrocyte phenotypes expressions at the 1st, 2nd and 4th weeks by using cytochemical staining technique, RT-polymerase chain reaction, and Western blotting methods.

Fedewa et al.[10] added ascorbic acid to RPMI in high amount as the MCCC. They observed that cartilage thickness decreased and modulus increased with changing of the RPMI media.

Kılıç et al.[32] studied the osteoblastic and chondroblastic differentiation potential of mesenchymal connective tissue (stromal) cells harvested from 10 healthy human bone marrow transplantation vendors between the ages of 4 months and 18 years. They emphasized the determination of the MCCC according to proliferation and differentiation of mesenchymal stromal cells including all sub-cultivation.

There are several important features that distinguish our study from other experimental studies published in the literature; 1) It is the first experimental study using human chondrocytes and comparing the culture media and the ITS premix added as additives, 2) The proliferation and differentiation effects were documented in the presence of the ITS premix solution to the most commonly available RPMI-1640 and DMEM, 3) This differentiation and reproduction occurred without the addition of a growth hormone to the MCCC.

In the present study, it was determined that ITS and DMEM increase the cell proliferation when used separately. The lowest viable cell number was observed in the DMEM and ITS premix solution group at the end of the 14th day and this might be defined as a toxicity of the ITS premix solution ingredient over primer chondrocyte culture, as a result of chemical reaction incompatible with DMEM. These results require further researches to be confirmed and repeat the importance of the MCCC and additives to the MCCC for chondrocyte proliferation.

Conflicts of Interest: No conflicts declared.

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