Middle-East Journal of Scientific Research 9 (1): 102-109, 2011
ISSN 1990-9233
© IDOSI Publications, 2011

In vitro Human Chondrocyte Culture; A Modified Protocol

[1,2]*Mahnaz Heidari,* [3]*Mohammad naghi Tahmasebi,* [1]*Samar Etemad,*
[4]*Sheida Salehkhou,* [4]*Hamed Heidari-Vala and* [1]*Mohammad Mehdi Akhondi*

[1]Reproductive Biotechnology Research Center, Avicenna Research Institute (ARI), ACECR, Tehran, Iran
[2]Nanobiotechnology Research Center, Avicenna Research Institute (ARI), ACECR, Tehran, Iran
[3]Orthopedic Department, Shariati hospital, Tehran University of medical sciences, Tehran, Iran
[4]Monoclonal Antibody Research Center, Avicenna Research Institute (ARI), ACECR, Tehran, Iran

Abstract: Cartilage tissue culture is a new biotechnological field that has been recently emerged to provide an alternative cell culture to the treatment of damaged human tissues such as liver, bladder, chondrocytes, etc. In this study, we investigated culture method and the general characteristics of chondrocytes derived from knee articular cartilage of patients. Main aim of present study was try and research for more applicable method with admissible efficiency level in order to chondrocyte culture and propagation. Human cartilage fragments were harvested by arthroscopic biopsy. Fragments were digested with enzyme. The chondrocytes obtained were then cultured in mixture of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) medium with concentration of fetal bovine serum and supplemented media with or without ITS, β TGF and ß FGF. The cells were kept for up to 4 weeks and were then characterized both morphologically and immunofluorescence studies (IHC). The results showed that after a few days, the cells began to change their appearance as polygonal to fibroblast like morphology. ITS supplementation provided several fold increase in chondrocytes compared to the standard-culture medium significant difference in the growth rates was shown using various growth media. IHC results showed that the collagen type II expression reduces after every passage and the collagen type I is expressed as early as passage one. This study has demonstrated that obtaining a sufficient number of human chondrocytes requires long duration process, current time-consuming procedure accelerated by above growth factors in human chondrocytes monolayer culture.

Key words: Articular cartilage · Chondrocytes · Cell culture · Growth factors

INTRODUCTION

The articular cartilage, also known as hyaline cartilage, is thin smooth glistening white tissue that covers the surface of all the diarthrodial joints in the human body. The chondrocytes are the only cells of the articular cartilage and are responsible for the production of the extracellular matrix. The articular cartilage injuries affect more than a million people in the world annually and surgery procedures are performed to treat these injuries [1]. The chondrocytes compose less than 5% wet weight and collagen makes up about 15-22% of the wet weight and contains 90-95% type II collagen fibers with a small percentage of types IX and XI [2, 3]. The joints diseases affect 500 million people around the world. The most noticeable lesions are trauma and osteoarthritis in conjunction with a large number of genetic or metabolic conditions such as acromegaly and hemophilia. The articular cartilage injuries may occur as a result of either traumatic mechanical destruction or progressive degeneration [3, 4]. The limited ability of the articular cartilage to self-repairing has lead to a wide variety of treatment procedures to varying levels of success [5]. The current treatment techniques range from cell-based therapy to regeneration of the chondrocytes and surrounding matrix for auto and allograft transplantation. Some other approaches include debridement of loose or impinging chondral flaps and stimulation of the intrinsic repair mechanism through subchondral bone [3].

Studies about human articular chondrocytes have been hampered because of the lack of availability and quality of the human articular cartilage and the difficulty in isolating and maintaining the human articular chondrocytes in culture. To date several culture systems

Corresponding Author: Mohammad Mehdi Akhondi, Reproductive Biotechnology Research Center, Avicenna Research Institute (ARI), ACECR, Tehran, Iran. Tel: +98-21-22432020.

have been used to maintain chondrocytes *in vitro* including monolayer cultures, suspension culture or spinner culture [6, 7]. In the present study, the articular cartilage samples from patients 20-30 years old were used. The validity of the age range was related to a study which reported that the cartilage culture from the cells showed no significant dependence on the age of the donor [8, 9]. It has been shown that when human chondrocytes are growing in monolayer cultures they proliferate, but they are hindered to express the specialized proteins of cartilage and become fibroblastic in appearance [10, 11]. This suggests that under these conditions, the chondrocyte phenotype is not stable. The expression of the human chondrocyte phenotype has been investigated either with freshly isolated cells or with passage for as many as six times *in vitro* [12, 13]. The previous study has shown that the knee joint is containing only a small amount of cartilage that can be harvested from lower weight-bearing surface at the donor site. This limitation scanty amounts of the isolated chondrocytes needs to be culture expanded in culture before a clinical treatment can be made. The number of chondrocytes can be obtained in monolayer culture system during short period of time with monolayer culture system. Chondrocytes cultured in monolayer have a tendency to dedifferentiate due to the cytoskeleton modification in two dimensional culture [14, 15].The combination of insulin transferrin selenium has been added in to the alginate beads culture system to increase the event of dedifferentiation in expanded chondrocytes [16].The regulatory effects of insulin transferrin selenium in human chondrocytes monolayer culture supplemented with serum and growth factors have not been investigated in previous studies. The proliferation of chondrocytes can also be accelerated by growth factors supplementation in the medium [17, 18]. The Various types of basal culture media supplemented with serum, Insulin Transferrin Selenium, Transforming Growth Factor, Basic Fibroblast Growth Factor have been widely used to provide basic nutrients for human chondrocytes. The culture medium plays an important role in chondrocyte growth and provides phenotypic stability chondrocytes [19]. The aim of this study was to improve the chondrocy culture teculture and evaluate cell preservation, proliferation and morphology.

MATERIALS AND METHODS

All tissue culture disposable materials (Petri dish, multiplate, flask, mesh 70) were purchased from falcon (Becton Dickinson AG, Switzerland).

All supplemented media and fetal bovine serum (FBS), medium culture (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) Media), antibiotics (Penicillin, Streptomycin, Gentamycin, Amphotericin B), enzyms (Trypsin, Collagenase, etc) were purchased from Gibco (life technologies, Switzerland). All the chemical and growth factor were of the highest grade and purchased from Sigma (England). In addition, All the Immunohistochemistry materials and monoclonal collagen I and II antibody were purchased from the Abcam (Germany).

Cartilage Biopsies: The human articular cartilage (internal femoral condyle) was obtained by surgery team in the Shariati Hospital from knee of 20-30 years patients that in their whom 20-30 years of age who attended arthroscopy for several reasons. The cartilage fragments had 1cm^2 area in diameter and were transported immediately after harvesting in sterile tubes containing phosphate buffered saline (penicillin/streptomycin and Amphotericin B).

Chondrocyte Isolation from Cartilage: Fragments of the articular cartilage were taken to the Avicenna Research Center and then enzyme digestion process was applied to isolate the cells according to the modified protocol of Archer *et al*. [9].

A sterile scalpel was used to cut the cartilage into small pieces and transferred to Petri dish. Sliced cartilages were maintained overnight at 37°C, 5% CO^2 as in ham's F12: DMEM containing fetal bovine serum, ascorbic 100 mg/ml and penicillin/streptomycin 100 u/100 mg/ml to ensure sterility of the specimens prior to enzymes digestion, the time of enzymatic digestion was adapted to the size of tissue fragments. The prepared cartilage was washed in phosphate buffered saline (PBS) containing antibiotics. The tissue was then incubated in the above growth medium containing trypsin (0.25% w/v) at 37°C, 5% CO^2 for 30 minutes. Supernatants were discarded and the cartilage was further digested in growth medium containing 0.8 mg/ml collagenase II for 4 hours at 37°C, 5%CO^2. The digested tissue was then allowed to settle and the supernatant containing the cells was removed and centrifuged at 1500 g for 5 minutes at 4°C. The final digest was centrifuged at 1500 g for 10 min. The cell pellet was washed three times in PBS or serum free medium. Cell suspensions were passed through a nitex nylon filter (mesh width 70mm) and re-suspended in serum containing medium. The cell viability was determined by trypan blue dye exclusion test 20 in hemocytometric chamber.

Table 1: supplements to the human chondrocytes culture medium

Group	Supplements
1	Ham's F12: DMEM + 2% FBS
2	Ham's F12: DMEM + 10% FBS
3	Ham's F12: DMEM + 1ng/ml βFGF + 1ng/ml TGFβ2+ 2% FBS
4	Ham's F12: DMEM + 1ng/ml βFGF + 1ng/ml TGFβ2+ 10µg /ml ITS
5	Ham's F12: DMEM + 1ng/ml βFGF + 1ng/ml TGFβ2+ 10µg/ml ITS + 2% FBS
6	Ham's F12: DMEM + 1ng/ml βFGF + 1ng/ml TGFβ2+ 10µg ITS + 10% FBS
7	Ham's F12: DMEM + 2% FBS+ ITS
8	Ham's F12: DMEM + 10% FBS+ ITS

Chondrocytes Monolayer Culture: The cells isolated cartilage were seeded in 4 well culture plates at low density (4000 cell/cm^2) in culture medium in an equal volume mix of ham's F12 medium and Dulbecco's modified eagle medium (F12:DMEM), supplemented with various factors in several study groups as shown above (Table 1).

Each culture mediums also contained Ascorbic acid (50µl/ml), penicillin (100µl/ml), streptomycin (100µl/ml), L-glutamine (200mM) and incubated in a CO^2 incubator at 5% CO^2, 37° C and saturated humidity for periods of up to 40 days. The culture medium was changed twice or three times per week.

Morphology and Passage Chondrocytes: The cell morphologic feature was examined daily by phase microscopy in an inverted microscope. The cultures were regularly photographed for monitoring cell morphology. When chondrocytes reached 70 to 90% confluency, the cells were detached mechanically, using trypsinization 1 to 2 min (0.25% w/v in Ca^{+2} and Mg^{+2} free PBS) and harvested chondrocytes were washed and re-suspended in PBS for, than total cell number and their viability was assessed using the trypan blue test.

Then cultured chondrocytes from each medium were passaged 3 times. Of course the consecutive applied medium and with the same in cell density and culture medium were similar to previous step. Eventually, the chondrocytes growth rate and viability cells in different groups were evaluated in different groups.

Assays of Cell Growth Properties: The growth rates of human chondrocytes grown ether as monolayers was measured by seeding cells at a density of 4×10^4 cell into 60mm dishes. About 24 hours after seeding and every 24 hours thereafter, for up to 12 to 16 days, cells from plates were harvested and counted with cell counter. The saturation density, that is the maximum number of cells per unit area of culture surface, was determined from cultures of chondrocytes that had reached the stationary phase of growth. The cells were fed every 3 to 5 days and stained 3 to 4 weeks later with trypan blue. To determine confluent cell monolayers cell were subcultured at 1:2 or 1:4 split ratios every 5 days to retain equivalence of population doublings with passages, a. 1:2 split was scored as population doubling and 1:4 split as 2 population doubling.

Immunofluorescence Studies Surveys: To verify the expression of types collagen I and II on the cells, chondrocytes was incubated with antibodies for types I and II at 1:500, 1:250, 1:100 concentration for one hour at 37°C after, than slides were washed 3 times in phosphate buffered saline (PBS) and incubated with 1/50 dilution (with PBS) of sheep anti mouse FITC in PBS as a secondary antibody under the same conditions as described for the primary antibody. Dapi at 2µg/ml was applied in some experiments with the secondary antibody for nuclear staining. Finally, the sections slides were washed three times in PBS and then observed directly on a fluorescence microscope.

Statistical Analysis: Statistical differences were performed by SPSS and Tukey-Kramer multiple comparison test. Differences were regarded as significant at $P<0.05$.

RESULTS

Chondrocytes Isolation: The chondrocytes were kept in culture from 10 to 40 days in order to reach at least one million cells. The total number of cell and viability were counted in culture. The mean wet weight of the human cartilage harvested was 100mg. The mean isolated chondrocytes yield was 2500 cells per mg of cartilage. The viability of the isolated chondrocytes was 98%.

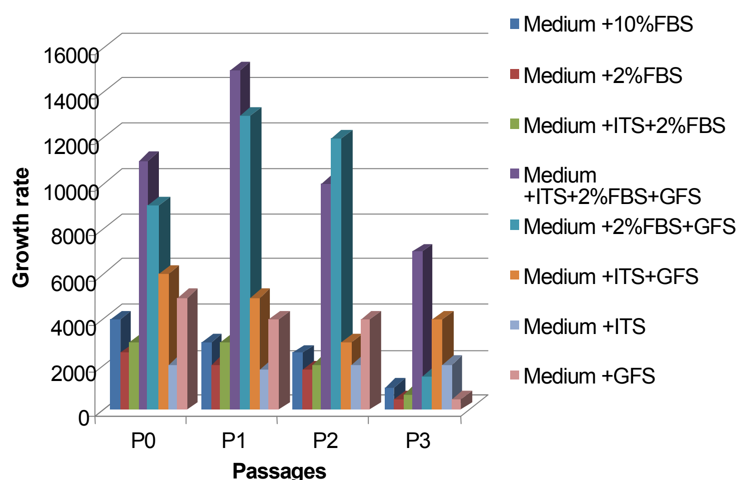

Fig. 1: Growth rate (average increase in cell number per day/cm2) of human cartilage chondrocytes cultured in medium supplemented with different factors at P0, P1,P2,P3 values were showed as mean + SEM (n=9), p<0.05.

Growth Characteristics of Cultured Human Chondrocytes: Several parameters of human chondrocytes culture such as growth rates, saturation densities and efficiencies were determined.

The saturation densities of the cells grown as in monolayers were also determined and found to be estimated $4{\times}10^5$ cells/cm2 as compared with human chondrocytes in the different medium cell culture. To determine the lifespan of the human chondrocytes, the cells were grown and passaged as in monolayer cultures until as long as they its entrance stopped dividing into stationary phase or failed to form confluent monolayers after about one 30 days with frequent of the growth medium. The lifespan of cultured cells was found to be in the range of 40-50 population doublings (Figure 1).

Morphology of Cultured Human Chondrocytes: After 40 days, the cells formed confluent monolayers and chondrocytes at passage one. The cells resembled polygonal morphology. However, the cells switched in to spindle morphology after serial passage.

The chondrocytes adhesion during cell culture resulted in change in the rounded shape characteristic of chondrocytes. The chondrocytes cultured in F12: DMEM supplemented with various factors, exhibited different morphologic feature in monolayer chondrocytes cultured in F12: DMEM with additional ITS showed minimal proliferation activity with only one or two cell doubling. The chondrocytes were relatively bigger and polygonal in shape on day 13 of primary culture. The chondrocytes in the medium stopped proliferation after weeks of primary culture, in this medium and was unable to reach confluence. The culture was terminated without further passage. The chondrocytes cultured in groups 1 and 8 were slow in proliferation and appeared polygonal in shape at primary culture (Figure 2, A-D).

Both culture reached about 60% confluency on day 15 of primary culture. The group 7 demonstrated higher cell densities compared to medium added with only 2% FBS chondrocyte culture. The primary cultures in media reached confluence on the second week and were able to be passaged until the third passage time. The chondrocytes appeared more elongated in shape at passage sixth and they were also spindle and fibroblastic. The chondrocytes cultured in F12: DMEM supplemented with only beta FGF and TGFβ B2 did were adhered on the culture plate, but were unable to proliferate.

The chondrocytes cultured in F12: DMEM + beta TGF+betaFGF+ITS supported better chondrocytes growth in primary culture and reached confluence on day 15 of the culture period. The chondrocytes in this medium appeared polygonal in shape appearance. The culture was polygonal shape and unable to grow beyond p1 and therefore culture was terminated.

The chondrocytes cultured in F12: DMEM +2% FBS + ITS + FGF + TGFβ medium adhered quickly to the culture plate and demonstrated high mitotic activity. The primary chondrocytes culture in media reached confluence on day 15 of culture and exhibited showed almost the same cell density. The chondrocytes usually maintained in high proliferation activity throughout the culture period until to the P3. The chondrocytes at P5 appeared slightly elongated in shape and remained small in size. Also, addition of this in the medium consisted of TGFβ and 2% FBS increased chondrocytes its growth rate. All the cultures in this study scored based on viability greater than 95% and no significant difference was noted among the various groups.

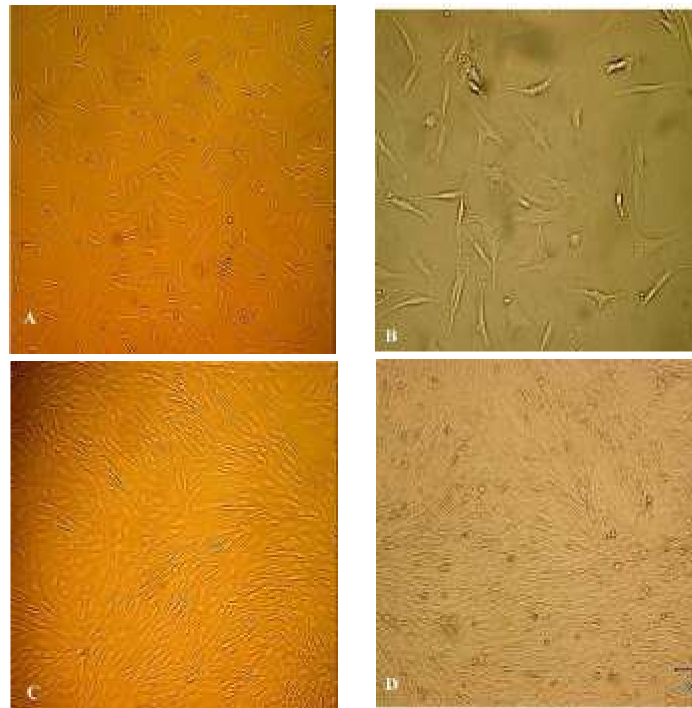

Fig. 2: Human chondrocytes primary culture (passage zero) after 40 days showed different morphology feature in DMEM/F12 supplemented with different factors

A: primary culture after 13 days B: primary culture after 25 days

C: culture of 40 days D: chondrocytes culture (passage 6)

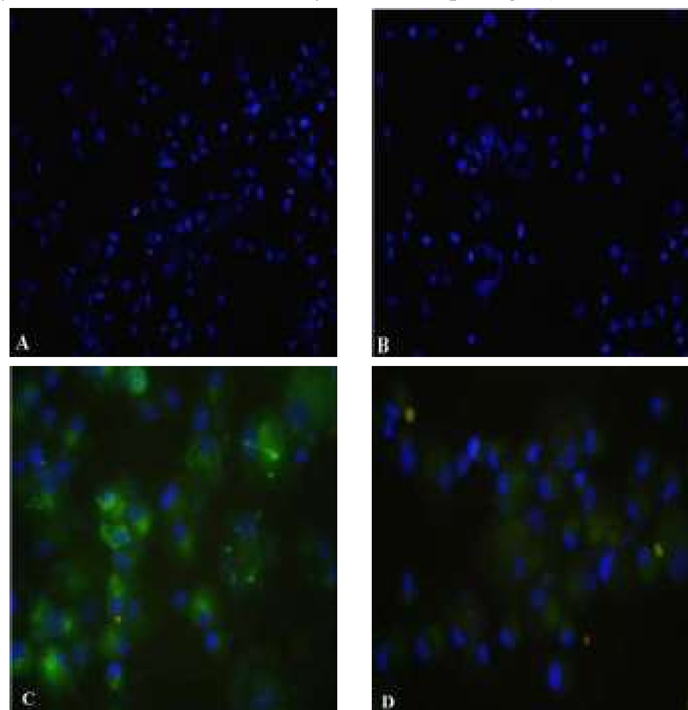

Fig. 3: Immunohistochemical staining of type I and II collagen on the monolayer cell showed positive staining in the microscope

A- Control negative reagent (TBS) B- Control negative (serum mice) C- Collagen I, 1/100 D- Collagen II, 1/250

Immunofluorescence Studies: In the process of chondrocyte culture it is important to ensure that expanded cell population retains its phenotypic function properties. The using immunohistochemical (IHC) staining chondrocytes. The chondrocytes culture in better medium supplemented showed higher expression of type II collagen in passages zero and first. On the other hand expression of type I collagen demonstrated in passages zero to sixth.

The staining was slightly positive at 4 weeks (Figure 3, A-D).

DISCUSSION

The number of cells available was not sufficient after passage zero expansion due to limited amount of tissue and needed to be further expanded in consecutive passages. This caused a reduction in the growth rate and hence took more time to reach the number required a favorite amount besides sacrificing the quality of chondrocytes obtained. The proliferation rate of chondrocytes decreases as alongside the age of donor [19]. For this in present study, the applicated cells from donors of up to 20-30 years of age old were used which and may have affected the results obtained. A methodology needs to be adopted in chondrocytes culture such as the addition of certain supplements to ensure proper maintenance of cells phenotypic express.

A culture medium which consists of including essential factors for the efficient propagation of human chondrocyte is necessary to make this technology more viable feasible for future clinical application. Supplementation of 2% FBS in the culture medium allowed chondrocyte to grow until passage 6. This minimum amount of serum has provided essential growth agents for chondrocytes to proliferate. A high serum culture medium (10% FBS) showed lower growth rate in cells chondrocytes. However, the addition of serum prompt chondrocytes to change its morphologic features to become more fibroblastic and differentiate in the monolayer culture. The effects of serum in on the chondrocytes culture medium was also reported by other studies[20-23]. The available insulin transferrin selenium used in this study was formulated for the supporting cell growth in low serum culture medium. Our results showed that supplementing culture medium was able to further enhance chondrocyte growth. The growth factors supplemented medium has provided sufficient number of chondrocytes for cartilage tissue formation [24, 25]. The chondrocytes culture in all experiment groups did not show a tendency to be hypertrophy in the monolayer culture.

In the present experiment, it has been shown that expansion of human articular chondrocytes in the presence of supplemental medium culture allows obtaining increase number of cells lead to sufficient amount of cells while retaining their chondrogenic capacity. Supplements increased the proliferation rate of chondrocytes and collagen type II expression in cells This is consistent with the previous study performed with bovine chondrocytes which were cultured on meshes [26,27]. These results indicated that mammalian chondrocytes share have common paradigms of response to growth factors, but with species-related specificity [28]. As it was mentioned earlier, the chondrocytes had polygonal morphology at passage zero and switched to spindle shape at passages 4 and 6. While articular chondrocytes morphology changed from polygonal in shape to be more elongated and bigger in size in passage 6. One logical reason for the fact that chondrocytes switching from polygonal morphology to spindle morphology after consecutive passages could be the arose from nature of culture. One of the other hand, some internal mechanisms change phenotypic expressions of the chondrocytes from expressing collagen gene type II toward expressing collagen gene type I [29]. The morphology of serially expanded chondrocytes changed from polygonal to become more elongated. At later passages, the chondrocytes seemed to adopt with many of the phenotypic traits of fibroblast like cell and synthesis more collagen type I rather than type II. This phenomenon has been reported for human nasal septum chondrocytes articular and articular chondrocytes [30, 31]. The chondrocytes cultured in different supplemented demonstrated no significant difference of in between collagen type I and II and the IHC staining patterns.

CONCLUSION

In the process of experimentation on cartilage tissue culture, it is important to ensure that the expanded cell population retains itself in a sustainable phenotypic function. The chondrocytes derived from articular cartilage biopsies have only a limited proliferative potential. They dedifferentiate upon repeated passaging and the number of cell division that the chondrocytes undergo *in vitro* decreases with age. The chondrocytes culture is feasible and safe technique in the laboratory and it is possible to use them for transplantation in clinical application as a surgical technique of articular cartilage regeneration. Present study could be a gleam of hope for

further studies to achieve a serum free medium formulating in human chondrocyte monolayer expansion and more reliable clinical indication.

ACKNOWLEDGEMENTS

This study was supported by the Avicenna Research Center (ARI) Grant. The authors are grateful for the advice and technical Immunohistochemistry support of Dr. Mahmood jeddi Tehrani and Dr. Amir Hassan Zarnani from the Department of Immunology ARI. Our sincere thanks are also to Dr. Ramin haji zargar, Dept. Orthopedic Shariati Hospital, Tehran University of Medical Sciences for providing human cartilage specimens.

REFERENCES

1. Brown, J.E. and T.P. Branch, 2000. Surgical alternative for treatment of articular cartilage lesions. J. Am. Acad Othop Surg., 8: 180-189.
2. Friedlaender, G.E. and V.M. Goldberg, 1991. The structure function and biologic repair of articular cartilage. In Bone and Cartilage Allografts: Biology and Clinical Applications, Eds., A. Ratcliffe and V.C. Mow, American Academy of Orthopaedic Surgeons , pp: 123-154.
3. Hunter, W., 1995. Of the structure and disease of articulating cartilages 1743. Clin. Orthop., 317: 3-6.
4. Buckwalter, J.A. and H.J. Mankin, 1997. Articular cartilage: degeneration and osteoarthritis, repair, regeneration and transplantation. J. Bone Joint Surg., 79: 612-632.
5. Frisbie, D.D., S.M. Bowman, H.A. Colhoun, E.F. DiCarlo, C.E. Kawcak and C.W. McIlwraith, 2008. Evaluation of autologous chondrocyte transplantation via a collagenmembrane in equine articular defects: results at 12 and 18 months. Osteoarthritis Cartilage, 16(6): 667-79.
6. Quinn, B., M.J. Costello, G. Dorange, J.G. Wilson and C. Mothersill, 2009. Development of an *in vitro* culture method for cells and tissues from the zebra mussel (Dreissena polymorpha). Cytotechnology, 59(2): 121-134.
7. Bartlett, W., C.R. Gooding, R.W. Carrington, J.A. Skinner, T.W. Briggs and G. Bentley, 2005. Autologous chondrocyte implantation at the knee using a bilayer collagen membrane with bone graft. A preliminary report. J. Bone Joint Surg Br., 87(3): 330-2.
8. Giannoni, P., A. Pagano, E. Maggi, R. Arbicò, N. Randazzo, M. Grandizio, R. Cancedda and B. Dozin, 2005. Autologous chondrocyte implantation (ACI) for aged patients: development of the proper cell expansion conditions for possible therapeutic applications. Osteoarthritis Cartilage, 13(7): 589-600.
9. Archer, C.W., J. McDowell, M.T. Bayliss, M.D. Stephens and G. Bentley, 1997. Phenotypic modulation in sub- populations of human articular chondrocytes *in vitro,* J. Cell Sci., 97: 361-71.
10. Paunescu, V., V. Poenaru Dan and D. Herman, 2004. Isolation and culture of human chondrocytes for autologous transplantion. TMJ., 54(1): 18-25.
11. Häuselmann, H.J., R.J. Fernandes, S.S. Mok, T.M. Schmid, J.A. Block, M.B. Aydelotte, K.E. Kuettner and E.J. Thonar, 1994. Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. J. Cell Sci., 107(1): 17-27.
12. Kolettas, E., L. Buluwela, M.T. Bayliss and H.I. Muir, 1995. Expression of cartilage-specific molecules is retained on long-term culture of human articular chondrocytes. J. Cell Sci., 108(5): 1991-9.
13. Bjornsson, S. And D. Heinegard, 1981. Isolation and culture techniques of fetal calf Chondrocytes. Biochem. J., 198(15): 141-8.
14. Loty, S., J.M. Sautier and N. Forest, 2000. Phenotypic modulation of nasal septal chondrocytes by cytoskeleton modification. Biorheology, 37(1-2): 117-25.
15. Lombello, C., J. Marins dos Reis and M. Cohen, 2003. Study on human chondrocyte culture viability for autologus transplantation in clinical application. Einstein, 1: 84-8.
16. Mandl, E.W., S.W. van der Veen, J.A. Verhaar and G.J. van Osch, 2002. Serum-free medium supplemented with high-concentration FGF2 for cell expansion culture of human ear chondrocytes promotes redifferentiation capacity. Tissue Eng., 8(4): 573-80.
17. Chua, K.H., B.S. Aminuddin, N.H. Fuzina and B.H. Ruszymah, 2005. Insulin-transferrin-selenium prevent human chondrocyte dedifferentiation and promote the formation of high quality tissue engineered human hyaline cartilage. Eur. Cell. Mater., 17: 58-67.
18. Olney, R.C., J. Wang, J.E. Sylvester and E.B. Mougey, 2004. Growth factor regulation of human growth plate chondrocyte proliferation *in vitro*. Biochem. Biophys. Res. Commun., 14: 317(4): 1171-82.

19. Barbero, A., S. Grogan, D. Schäfer, M. Heberer, P. Mainil-Varlet and I. Martin, 2004. Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. Osteoarthritis Cartilage, 12(6): 476-84.
20. Choi, Y.C., G.M. Morris, F.S. Lee and L. Sokoloff, 1980. The effect of serum on monolayer cell culture of mammalian articular chondrocytes. Connect Tissue Res., 7(2): 105-12.
21. Hu, D.N., P.Y. Yang, M.C. Ku, C.H. Chu, A.Y. Lim and M.H. Hwang, 2002. Isolation and cultivation of human articular chondrocytes Kaohsiung. J. Med. Sci., 18(3): 113-20.
22. Gerber, I. and I. Gwynn, 2001. Influence of cell isolation, cell culture density and cell nutrition on differentiation of rat calvarial osteoblast-like cells *in vitro*. Eur. Cell. Mater., 24(2): 10-20.
23. Bujía, J., M. Sittinger, E. Wilmes and C. Hammer, 1994. Effect of growth factors on cell proliferation by human nasal septal chondrocytes cultured in monolayer. Acta Otolaryngol., 114(5): 539-43.
24. Bujía, J., M. Sittinger, P. Pitzke, E. Wilmes and C. Hammer, 1993. Synthesis of human cartilage using organotypic cell culture. J. Otorhinolaryngol. Relat Spec., 55(6): 347-51.
25. de Haart, M., W.J. Marijnissen, G.J. van Osch and J.A. Verhaar, 1999. Optimization of chondrocyte expansion in culture. Effect of TGF beta-2, bFGF and L-ascorbic acid on bovine articular chondrocytes. Acta Orthop Scand., 70(1): 55-61.
26. Martin, I., R. Suetterlin, W. Baschong, M. Heberer, G. Vunjak-Novakovic and L.E. Freed, 2001. Enhanced cartilage tissue engineering by sequential exposure of chondrocytes to FGF-2 during 2D expansion and BMP-2 during 3D cultivation. J. Cell Biochem., 83(1): 121-8.
27. Jakob, M., O. Démarteau, D. Schäfer, B. Hintermann, W. Dick, M. Heberer and I. Martin, 2001. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation *in vitro*. J. Cell Biochem., 26: 81(2): 368-77.
28. Giannoni, P., A. Crovace, M. Malpeli, E. Maggi, R. Arbico, R. Cancedda and B. Dozin, 2005. Species variability in the differentiation potential of *in vitro*-expanded articular chondrocytes restricts predictive studies on cartilage repair using animal models. Tissue Eng., 11(1-2): 237-48.
29. Kusuhara, H., N. Isogai, M. Enjo, H. Otani, Y. Ikada, R. Jacquet, E. Lowder and W.J. Landis, 2009. Tissue engineering a model for the human ear: assessment of size, shape, morphology and gene expression following seeding of different chondrocytes. Wound Repair Regen., 17(1): 136-46.
30. Nur Adelina, A.N., B.S. Aminuddin, S. Munirah, K.H. Chua, N.H. Fuzina, L. Saim and B.H. Ruszymah, 2004. Phenotypic expression of collagen type II and collagen type I gene in monolayer culture of human auricular chondrocytes. Med. J. Malaysia, 59: 188-9.
31. Isogai, N., H. Kusuhara, Y. Ikada, H. Ohtani, R. Jacquet, J. Hillyer, E. Lowder and W.J. Landis, 2006. Comparison of different chondrocytes for use in tissue engineering of cartilage model structures. Tissue Eng., 12(4): 691-703.