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Study of Carbon Nano-Tubes Effects on the Chondrogenesis of Human Adipose Derived Stem Cells in Alginate Scaffold

Ali Valiani, Batool Hashemibeni, Ebrahim Esfandiary, Malek Masoud Ansar[1], Mohammad Kazemi, Nafiseh Esmaeili[2]

Department of Anatomical Sciences and Molecular Biology, Medical School, Isfahan University of Medical Sciences, Isfahan, I.R. Iran, [1]Department of Anatomical Sciences, Medical School, Guilan University of Medical Sciences, Guilan, I.R. Iran, [2]Department of Immunology, Medical School, Isfahan University of Medical Sciences, Isfahan, I.R. Iran

Correspondence to:
Dr. E. Esfandiari
Department of Anatomical Sciences and Molecular Biology, Medical School, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.
E-mail: esfandiary@med.mui.ac.ir

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Original Article

ABSTRACT

Background: Osteoarthritis is one of the most common diseases in middle-aged populations in the World and could become the fourth principal cause of disability by the year 2020. One of the critical properties for cartilage tissue engineering (TE) is the ability of scaffolds to closely mimic the extracellular matrix and bond to the host tissue. Therefore, TE has been presented as a technique to introduce the best combination of cells and biomaterial scaffold and to stimulate growth factors to produce a cartilage tissue resembling natural articular cartilage. The aim of study is to improve differentiation of adipose derived stem cells (ADSCs) into chondrocytes in order to provide a safe and modern treatment for patients suffering from cartilage damages.

Methods: After functionalization, dispersions and sterilizing carbon nano-tubes (CNTs), a new type of nanocomposite gel was prepared from water-soluble CNTs and alginate. ADSCs seeded in 1.5% alginate scaffold and cultured in chondrogenic media with and without transforming growth factor-β_1 (TGF-β_1) for 7 and 14 days. The genes expression of sex determining region Y-box 9 (SOX9), types II and X collagens was assessed by real-time polymerase chain reaction and the amount of aggrecan (AGC) and type I collagen was measured by ELISA.

Results: Our findings showed that the expression of essential cartilage markers, SOX9, type II collagen and AGC, in differentiated ADSCs at the concentration of 1 μg/ml CNTs in the presence of TGF-β_1 were significantly increased in comparison with the control group ($P < 0.001$). Meanwhile, type X collagen expression and also type I collagen production were significantly decreased ($P < 0.001$).

Conclusions: The results showed that utilized three-dimensional scaffold had a brilliant effect in promoting gene expression of chondrogenesis.

Keywords: Adipose derived stem cell, alginate, carbon nano-tube, chondrogenesis, transforming growth factor-β_1

INTRODUCTION

Osteoarthritis (OA) is one of the most common diseases in

middle aged populations of the World and could become the fourth principal cause of disability by the year 2020.[1-4] The limited capacity for self-regeneration and lack of decisive treatment has prompted related researches into tissue engineering (TE), which combine chondrogenic cells, scaffold materials, and growth factors.[5]

In recent 2 decades, TE has presented modern techniques for treatment of acute traumatic OA including autologous chondrocyte culture and transplantation,[6] with brilliant results and also differentiation of adult stem cells into chondrocyte which is still being studied in order to result in transplantation in synovial joints of patients. Adipose derived stem cells (ADSCs) is an easily accessible source for adult stem cells which is obtained from subcutaneous fat and has the potentiality to differentiate into various cell types including chondrocytes, osteoblasts, adipocytes, and myocytes.[7,8] In articular cartilage, chondrocytes are surrounded by an abundant extracellular matrix (ECM), which is composed of a highly hydrated complex network of molecules.[5] ECM plays a crucial role in cell–substrate adhesion, proliferation, and migration.[9] In cartilage TE, scaffold is necessary because it can somehow perform as ECM *in vivo*. This scaffold should have the properties of biocompatibility and biodegradability with porosity characteristics.[10,11]

The required scaffold for repairing damaged cartilage could be replaced by a natural matrix which provides stability and integration, similar to the host tissue ECM, so host cells could be replicated. Alginate hydrogels are derived from natural polysaccharide, which is soluble in water and is chemically similar to glycosaminoglycans (GAG). Studies showed that the alginate gel provides a uniform distribution of the chondrocytes and maintains their differentiated phenotype.[12]

Studies show that physical factors can also improve the process of stable cartilage formation.[13,14] For example, hydrostatic pressure imposed on the differentiation of stem cells reduces the expression of hypertrophic markers.

In 21st century, nanotechnology has emerged as a core technology to enhance and improve health and life quality.[15] Therefore, nano-materials such as carbon nano-tubes (CNTs) have been widely used in medical applications and have improved diagnostic methods, drug delivery, and regenerative medicine.[16,17] In particular, CNTs are one of the nano-material representatives which are reported by Somia Ijima in 1991.[18] CNTs are formed by carbon atoms which are derived from difference sources such as graphite.[19] CNTs are of two principle types, can be composed of a single tube called single wall and concentric cylinders of carbon called multi wall.[20] These materials have excellent chemical and physical properties such as high mechanical strength, ultra-light weight, high surface area, rich electronic conductivity, thermal stability, and high capillary action properties.[21] Although extensive studies made on physical, electrical, and chemical properties of CNTs, a little research has been done on their biological and biomedical applications particularly TE.[19,22]

Since CNTs are used for strengthening of mechanical polymers and composites, to improve the mechanical properties of alginate, they can also used as a reinforcing material to fabricate a nano-composite scaffold.[23] Similar to microtubules, CNTs are also highly resilient[24] and are similar to bundles of collagen fibers in connective tissue. CNTs films are especially attractive because they appear to be biologically inert, an important reason of their possible usefulness in biomedical applications.[25] Recent studies have expressed that purification of CNTs with carboxylic groups causes higher solubility and biocompatible properties in them and also reduces their toxicity.[26] Despite concerns, *in vivo* and *in vitro* studies of their usage as nano-materials have been investigated.[20]

Zhang *et al*. have reported that effect of CNTs on the proliferation and differentiation of osteoblasts is time- and dose-dependent, in concentration of 50 μg/ml expression of $Runx_2$ and Col I are decreased, and in 0.1 and 1 μg/ml proliferation of cells is excellent.[27]

Yildirim *et al*. stated that cell adhesion and proliferation of endothelial cells in composite CNTs and Alginate scaffold is improved.[23] Chen *et al*. have reported that Rosette nano-tube/hydro-gel composites promote synoviocyte fibroblast-like type B cell adhesion, viability, and chondrogenic differentiation compared to hydrogels without RNTs.[28] Furthermore, Spadaccio *et al*. showed that Poly L lactic Acid/hydroxyapatite nano-composites induce differentiation of human mesenchymal stem cells in achondrocyte-like phenotype with generation of a proteoglycan based matrix.[29]

Although stem cells differentiation into chondrocytes has been carried out various natural and synthetic scaffolds, CNTs has not been used as scaffolds using in cartilage TE yet. Considering advantages of using CNTs as scaffolds in cell culture on one side, and limited experiences of using CNTs in chondrogenesis on the other side, made us to investigate the effect of CNT/alginate hydro-gel composite on ADSCs while our medium was enriched with transforming growth factor-β_1 (TGF-β_1). The aim of this study is to improve differentiation of ADSCs into chondrocytes in order to create a safe and modern treatment for patients suffering from cartilage damages.

METHODS

Functionalizing and dispersions of carbon nano-tubes

Carbon nano-tubes were obtained from Nano-structure and Amorphous Material Co. (Product No. 1281 YJS) with average outside diameter 1.1 nm and 0.5-2 µm length. CNTs were not dispersible in deionized (DI) water or alcohol even after sonication. This suspension agglomerated quickly and settled down in the bottle. For preventing agglomeration, we had to functionalize CNTs. Therefore, 30 mg of pure CNTs was suspended in 60 ml of a 1:1 mixture of sulfuric acid 98% and nitric acid 70% (Merck) in a round bottom flask equipped with a condenser and was refluxed for 1 h. Next, the refluxed solution was centrifuged for 10 min and the supernatant was removed and diluted with 1200 ml of DI and was collected on a filtering membrane. Finally, the sample on filtering membrane was dried in a vacuum chamber at 80°C overnight.[30,31] The CNTs preparation dispersed in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) using a sonication/centrifugation protocol described by Chin *et al*.[31] Briefly, 1 mg of CNTs-containing powder was dispensed into an eppendorf tube containing 1 ml of DMEM/FBS, vortexes for about 1 min, and probe sonicated for 10 min at 0°C. The resulting black suspension was centrifuged in an eppendorf tube (2 min at 16,000 g). The supernatant was carefully recovered to afford DMEM–CNTs dispersion. Functionalizing and dispersions of the CNTs was determined by transmission electron microscopy (TEM) and Fourier transform-infrared (FT-IR) spectroscopy.

Isolation and proliferation of adipose derived stem cells (cell harvesting and culture of adipose derived stem cells)

Subcutaneous adipose tissue samples were collected in falcon having phosphate-buffered saline (PBS) from four patients (30–50 year) who filled the consent form before undergoing cesarean section or abdominal surgery. All samples were digested with 0.075% collagenase type I (Sigma) and incubated for 30 min at 37°C in the lab. Next, DMEM low glucose (LG) (Sigma) containing 10% FBS (Invitrogen) was added for enzyme inactivation before being centrifuged (1200 rpm, 15 min). Removing supernatant, cultured cell pellet in 25 cm^2 flasks with DMEM LG, 10% FBS, 1% penicillin and streptomycin (Gibco) and incubated with 5% CO_2, 37°C. The medium was changed every 4 days. When the cells reached 80% confluence, detached with 0.05% trypsin/0.53 mM ethylenediaminetetraacetic acid (Sigma) and passage P3-p5 cells were seeded in CNTs/alginate scaffold [Figure 1].

Scaffold fabrication

After CNTs functionalizing and dispersion, the CNTs suspension was sterilized by autoclaving (Napco TM Autoclave VA, USA) for 20 min at 118°C.[30,33,34] The alginate solution was prepared by dissolving alginate powder (Sigma) in sodium chloride (0.9%) as a 1.5% (w/v) solution. To

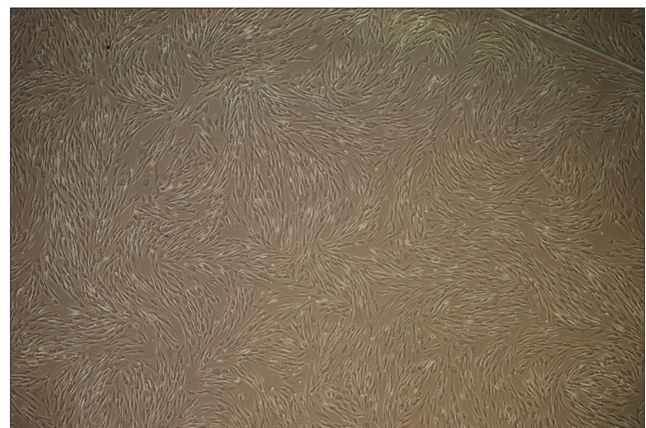

Figure 1: Inverted microscopic images of monolayer adipose derived stem cells at passage tow (magnification ×60)

eliminate the contaminant, the alginate solution was passed through a filter with a pore size of 0.2 µm. 0.1 and 1 µg/ml of aseptic CNTs suspension was mixed with 2 ml of sterile alginate solution and the mixture was then stirred for 24 h using a magnetic stirrer.[23]

Encapsulation and culture procedure in alginate

Adipose derived stem cells (p3-p5) were separately resuspended in 1.5% alginate/CNTs at 5×10^6 cells/ml. The alginate/CNTs/cell suspension was expressed through a 23-gauge needle into a 102 mM $CaCl_2$ solution (Merck). The alginate beads after 15 min were washed twice in 0.9% saline solution and once in DMEM-high glucose (HG) (Gibco), and finally 2 ml chondrogenic media was added to each well of 12-well [Figure 2]. Chondrogenic culture media contains DMEM-HG (Gibco), penicillin and streptomycin 1% (Gibco), dexamethasone 10^{-7} M (Sigma), ascorbat-2-phosphate 50µg/ml (Sigma), bovine serum albumin 1% (Sigma), linoleic acid 5 µg/ml (Sigma), insulin-transferrin-selenium 1% (Sigma), with and without adding TGF-β_1 10 ng/ml (Sigma). The plates were incubated in 5% Co_2 at 37° and were replaced every 4 days. Supernatant mediums on days 7 and 14 were frozen at –20°C for ELISA.

Elisa

Aggrecan (AGC) and type I collagen which were produced in supernatant media with and without TGF-β_1 on days 7 and 14 were measured by ELISA. AGC kit and type I collagen was respectively purchased from Invitrogen and MD bioproducts according to their manufacturers protocols. Finally, samples were read in ELISA reader (Hyperion, Microreader 4 plus) with 450 nm wave length.

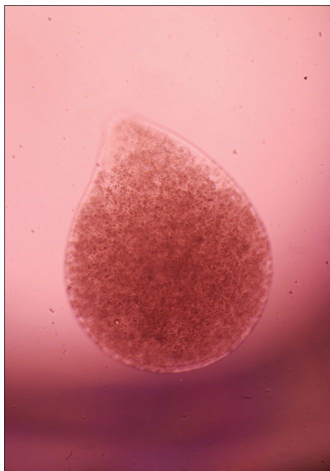

Figure 2: Inverted microscopic images of adipose derived stem cells seeded in alginate/carbon nano-tubes beads. Note the spherical shape of the cells within the bead (magnification ×60)

Real-time polymerase chain reaction (PCR)

At first, alginate beads on 7th and 14th days were washed with PBS and they were placed in 1.5% 55 mM citrate sodium (Sharlau) and 0.15 mM NaCl (Merck) for digestion. The resultant solution was centrifuged for 10 min at 1200 rpm and the derived cell pellet was used for the ribonucleic acid (RNA) extraction with RNeasy mini kit (Qiagen, Cat. No. 74101).[35] For lysing cells, first, a 990 µl Trizol (Invitrogen) and 10 µl of 2-mercaptoethanol (Sigma) solution was mixed and kept in room temperature (RT) for 5 min. Next, 200 µl chloroform was added and shacked firmly for 15 s and was kept in RT for 2-3 min and centrifuged for 15 min at 4° at 12,000 g. The supernatant aqueous phase was transferred to 1.5 ml microtube and the same volume of ethanol 70% was added and mixed. The resultant solution was put into columns in the kit and the rest of instruction was carried out according to kit protocol in which RNase free DNase set (Qiagen) applied for elimination of possible deoxyribonucleic acid (DNA) contamination. The extent of derived RNA was measured by spectrophotometer (Biophotometer, Eppendorf) at 260/280 nm wave length. Reverse transcription for complementary deoxyribonucleic acid (cDNA), 100 ng RNA used by recruitment of RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's protocol.

Relative quantification of the expression sex determining region Y-box 9 (SOX9) types II and X collagens was measured using Maxima SYBR® Green/Roxq PCR master Mix 2X (Fermentas), with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer as internal control. The calculation was performed via comparative Ct (ΔΔ Ct). The reactions conducted in 20 µl with, 10 µl SYBR® Green, 7.5 µl H_2O, 0.25 µM forward and reverse primers, and 1.5 µl cDNA as planned by Step One Plus real-time PCR system (Applied Biosystem) in following: Primary

denaturation in 95° for 10 min, denaturation in 95° for 15 s, Annealing and Extension in 60° for 1 min–the whole process was done in 40 cycles-and finally melt curve was depicted. All experiments were done triplicate for each specimen. The applied primers for real-time PCR were designed by Allele ID 7.6 software as indicated in Table 1.

Statistical analysis

The Kolmogorov-Simonov test was used for assessing the normal distribution of variables and (one-way analysis of variance) with the least significant difference *post-hoc* test was used for comparison of the ELISA and real-time PCR results in different time and groups.

RESULTS

Characterizations and dispersions of carbon nano-tubes

Microscopic analyses

Transmission electron microscopy images of the reflux functionalized CNTs bundles are shown in Figure 3. Based on the TEM results obtained from more dilute dispersions in methanol, it is evident that the bundles are weakly held together, as the images show large numbers of individual tubes and thin bundles.

Fourier transform-infrared spectroscopy

Fourier transform-infrared spectra of pristine and reflux functionalized CNTs were obtained to determine the structure of the chemical groups form on the nano-tube sidewalls and tube ends. The FT-IR spectrum [Figure 4] showed a number of infrared peaks, which were assigned as following: The peak at 3600-3200/cm was due to carboxylate O–H stretching and 2960-2930/cm was assigned to aliphatic C–H stretching. Furthermore, the peak at 1620-1450/cm was due to carboxylate O–H bending. The peaks at 1162 and 1114/cm were due to C–O stretches and C–H bending. The peaks at 1629 and 1717/cm may also be linked to carboxylate C = O or to aromatic C = C stretches. The FT-IR was performed on aFT/IR-6300 (400-4000/cm), JASCO, Japan.

Table 1: The genes and primer sequences used for real-time PCR

Gene name	Primer sequences	Size (base pair)
SOX9-F	TTCAGCAGCCAATAAGTG	133
SOX9-R	GTGGAATGTCTTGAAGGTTA	
Col II-F	CTGGTGATGATGGTGAAG	130
Col II-R	CCTGGATAACCTCTGTGA	
Col X-F	AGAATCCATCTGAGAATATGC	187
Col X-R	AGAATCCATCTGAGAATATGC	
GAPDH-F	AAGCTCATTTCCTGGTATG	125
GAPDH-R	CTTCCTCTTGTGCTCTTG	

F=Forward primer, R=Reverse primer, SOX9=Sex determining region Y-box 9, Col II=Collagen type II, Col X=Collagen type X, GAPDH=Glyceraldehyde 3-phosphate dehydrogenase, PCR=Polymerase chain reaction

Gene expression of sex determining region Y-box 9, type II and X collagens

Temporal expression of genes (total messenger ribonucleic acid [mRNA]) involved in chondrogenesis was extracted from alginate/CNTs beads at different culture times and groups. The mRNA of different genes was measured and normalized to the expression of housekeeping GAPDH. The mRNA expression of SOX9, types II and X collagen was detected in all groups and some significant differences were found in some time points [Figure 5].

The results of real-time PCR disclosed that time (on days 7 and 14) and either absence or presence of TGF-β_1, as well as CNTs, have an effect on expression of SOX9 gene ($P \leq 0.001$). Expression of SOX9 on day 14, particularly in the presence of TGF-β_1, was significantly more than the expression on 7th day. Expression of SOX9 in the presence of TGF-β_1 significantly increased in comparison with the absence of TGF-β_1. There were no difference between expression of SOX9 in the absence of TGF-β_1 at the concentration of 1 μg/ml CNTs and the control group, though, at the concentration of 1 μg/ml there was a significant increase in the TGF-β_1 treated group. In contrast, in the absence of TGF-β_1, expression of SOX9 at the concentration of

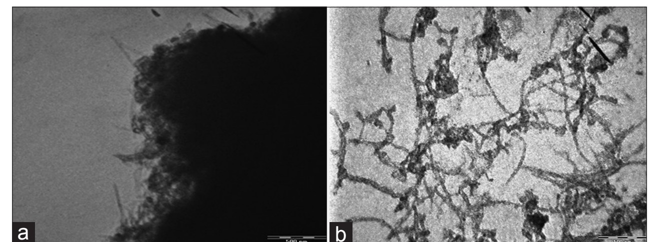

Figure 3: Transmission electron microscopy images of carbon nano-tubes, (a) Pristine; (b) After mixed acid reflux

0.1 μg/ml had a significant increase comparing with the control group. Whereas, at the concentration of 1 μg/ml, the expression of SOX9 was significantly decreased [Figure 5a].

Furthermore, all three variables (time, TGF-β_1 and CNTs) have effects on the expression of type II collagen gene ($P \leq 0.001$). In the presence of TGF-β_1, expression of type II collagen on day 14 was greater than day 7 and in contrary, in the absence of TGF-β_1 expression type II collagen on 14th day was less than on 7th day. The expression of type II collagen in the presence of TGF-β_1 was significantly greater than the case without TGF-β_1. With TGF-β_1 and at a concentration of 0.1 μg/ml of CNTs, expression of type II collagen was significantly desirably higher than the control group and at a concentration of 1 μg/ml CNTs, the similar results were observed [Figure 5b].

The results of type X collagen gene revealed that the time (days 7 and 14) does not effect type X collagen expression ($P = 0.42$). However, either absence or presence of TGF-β_1 as well as CNTs effects on the expression of type X collagen ($P \leq 0.001$). In the presence of TGF-β_1, expression of type X collagen is dropped. Comparing with the control group, type X collagen expression at the concentration of 0.1 μg/ml CNTs is decreased. Moreover, the expression of type X collagen is similar at both concentrations (0.1 and 1 μg/ml) [Figure 5c].

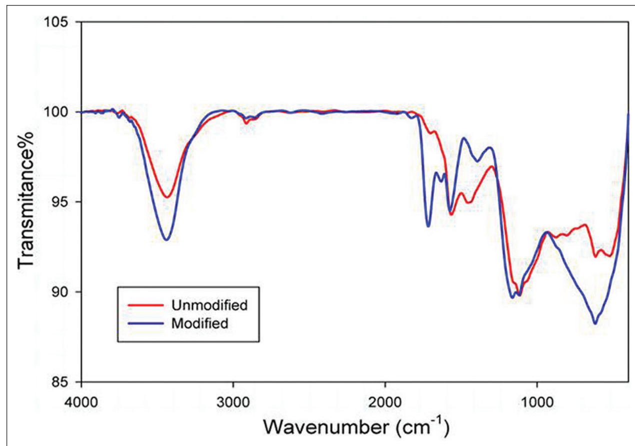

Figure 4: Comparison of the Fourier transform-infrared spectra for pristine (red) carbon nano-tubes and after mixed acid reflux (blue)

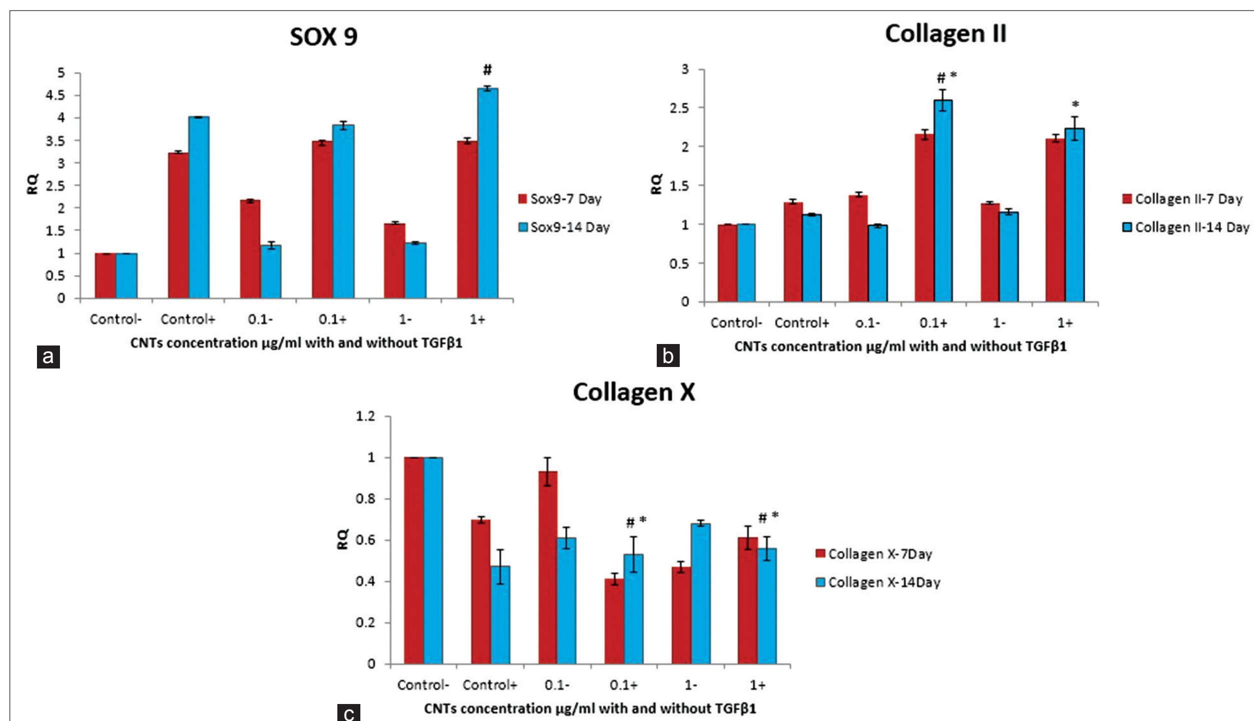

Figure 5: Chondrogenic-related gene expression of sex determining region Y-box 9 (a); type II collagen (b) and type X collagen (c) of alginate/carbon nano-tubes beads with and without transforming growth factor-β1 (TGF-β1) at different culture times (7 and 14 days) and groups relative to the housekeeping GAPDH. Values are the means ± standard deviation of triplicate experiments. Asterisks indicate that these groups statistically significant with TGF-β1 groups than those groups without TGF-β1. Symbol (#) indicate that this group with TGF-β1 is statistically significant in relation to control group ($P < 0.001$)

Production of aggrecan and type I collagen

Aggrecan, and type I collagen was evaluated by ELISA. According to results, the statistical analysis showed that the time (days 7 and 14) and absence or presences of TGF-β_1 as well as CNTs effect type I collagen production ($P \leq 0.001$). There were not any significant differences between CNTs (at the concentration of 0.1 μg/ml) and control group in type I collagen production; however, at the concentration of 1 μg/ml, production of type I collagen was significantly decreased [Figure 6a].

Moreover, the AGC production during (on days 7 and 14) with or without TGF-β_1 significantly effect ($P \leq 0.001$). The mean of AGC production on day 14 increased significantly but was not on 7th day. Furthermore, in the presence of TGF-β_1, AGC was produced more significantly than the absence of TGF-β_1. The mean difference between 0.1 and 1 μg/ml concentrations of CNTs was not statistically significant [Figure 6b].

DISCUSSION

One of the critical properties for cartilage TE is the ability of scaffolds to closely mimic the ECM and bond to the host tissue.[28,29] Since natural articular cartilage usually has a low cell density with little self-regeneration ability,[36,37] it is critical to implant enough cells to maintain relatively high cell viability for cartilage defect repair. In recent years, researchers have been studying viability, proliferation, and differentiation of stem cells in different scaffolds with CNTs but there is no detailed report about chondrogenesis in alginate/CNTs scaffold.

In this paper, a new type of nano-composite gel was prepared from water-soluble CNTs, and alginate hydro-gel. Alginate gels are able to controlled release of growth factors and cytokines.[38] Further, we expected the incorporation of soluble CNTs into alginate hydro-gel to increase the cross linking density between polysaccharide chains. The CNTs have significantly higher ability in adsorbing proteins because their larger surface area provides larger amount of protein sorbent and the diameter of the CNTs is similar with the protein dimension, and finally, nanostructure surfaces promote the formation of protein aggregates.[39,40] In fact, the biocompatibility and biodegradability properties of nano-composite scaffolds are critically important in TE applications. Not only should the engineered biomaterial be cytocompatible, but it should also be biodegradable over a desired period of time. Because alginate hydro-gels are known to be biocompatible and biodegradable as well as readily available, they were selected to be used in this study.[41,42]

To date, functionalizing CNTs for propose of biomedical applications and chemical surface modification of CNTs (chemical-treated) shows more hydrophilic behaviors and better dispersibility in comparison with CNTs sonication alone. This may be due to the OH groups formed in the acid-washed case that makes hydrogen bind with water molecules.[40,43] In this study, we employ TEM and FT-IR to describe the raw and functionalized CNTs samples. A typical FT-IR spectrum of CNTs treated with acidic mixture (H_2SO_4/HNO_3) for 1 h shows that a new peak appears around 1717/cm. It is normally assigned to the C = O strength vibration in the COOH group, which means that the acid-mixture treatment will introduce some C = O groups

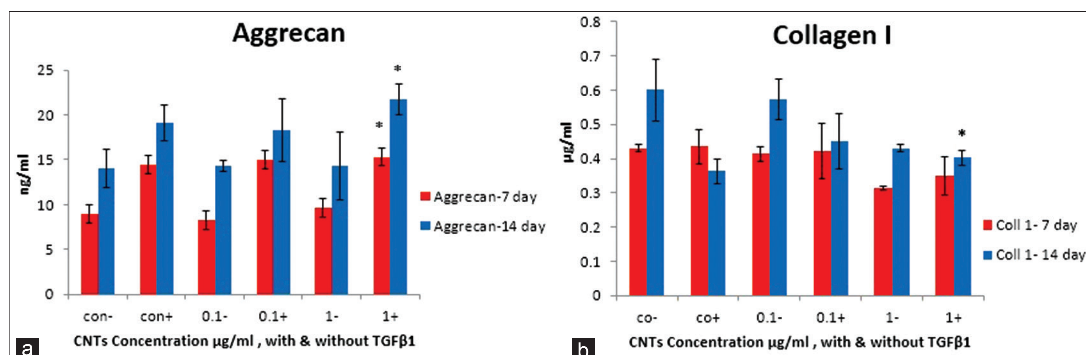

Figure 6: The results of ELISA analysis for aggrecan (a) and type I collagen (b) in supernatant media with and without transforming growth factor-β1 (TGF-β1) on days 7 and 14. Values are the means ± standard deviation of triplicate experiments. Asterisks indicate that this group with TGF-β1 is statistically significant in relation to control group ($P < 0.001$)

at the end or side of the CNTs. Meanwhile, the hydrogen binding formation between the-COOH groups became more effective. We also found that initially, in the treated CNTs, the peak is around 1564/cm, which is assigned to the C = C groups; its shift to a higher frequency around 1574/cm, may suggest a change (oxidation) in the structure of the CNTs. Zhang, *et al*.,[43] indicates the effect of chemical oxidation on the structure of SWNTs by using different oxidants, which is consistent in our findings. Furthermore, in this study, we investigated differentiation of chondrocytes from ADSCs in alginate/CNTs scaffold with and without TGF-β_1 for 7 and 14 days.

According to our findings, progressive increase of genes expression of SOX9 and type II collagen during 14 days and also existence of AGC in supernatant media as essential cartilage markers in alginate/CNTs scaffolds, confirms that ADSCs is differentiated to chondrocytes. Comparing investigation results of markers after 2 weeks indicated that in addition to growth factors (TGF-β_1), the presence of CNTs in alginate scaffolds has also contributed to this improvement.

ELISA results indicated that differentiated ADSCs in alginate/CNTs scaffolds with TGF-β_1 has significantly higher AGC production than control one on day 14 ($P < 0.001$). This is in agreement with recent findings of Chen *et al*. They demonstrated that SFB cell viability and chondrogenic differentiation with RNTs hydro-gel composites had higher GAG content per unit DNA compared to controls.[28] Because the highest proportion of ECM is produced at the above mentioned time, the day 14 is the most important time point in chondrogenesis *in vitro*.[44,45]

Types I collagen is considered as a negative marker in chondrogenesis, and unfortunately, MSCs especially ADSCs and bone marrow (BM)-MSCs produce a lot of type I collagen,[46,47] which is not appropriate in the case of implantation and after *in vivo* implantation, fibrocartilage is produced instead of hyaline one.[48] Contrary to this fact, scaffolds that maintain spherical shape of cells and prevent their contact to each other like agarose and alginate, basically enhance chondrogenesis and inhibit the production of collagen type I.[49] Observations done on day 14 revealed a decrease of collagen I in the group of 1 μg/ml CNTs comparing with the control group which is consistent with Yang *et al*. stated that collagen I in BM-MSCs decreases during chondrogenic differentiation in alginate and pellet culture.[12]

The adsorption of proteins depends significantly on surface nanostructure and that the relevant morphological parameters. Comparing with the control group, alginate/CNTs scaffolds has significantly higher ability in adsorbing proteins[39] as Nayak *et al*. showed that the CNTs had not any cytotoxicity and they could accelerate cell differentiation to form cartilage, by creating a more viable microenvironment for the stem cells.[50] In the other words, the CNTs may modulate the quantity and conformation of the adsorbed proteins and consequently modulate not only the adhesion of cells but also the differentiation of the cells toward the chondroblastic lineage.

Real-time-PCR analysis of ADSCs cultured at 7th and 14th days in alginate/CNTs scaffolds and chondrogenic medium with TGF-β_1 showed increased expression of chondrogenic-related genes, collagen type II and SOX9 in the treatment groups compared with control ones [Figure 5b], which is in agreement with recent findings of Fong *et al*. They demonstrated enhanced cell attachment, cell proliferation, and gene expression of SOX9 and collagen II on human Whartons Jelly stem cells on polycaprolactone/collagen nanoscaffolds in the presence of chondrogenic medium for 7, 14, and 21 days.[51] Type II collagen was localized extracellular while SOX9, which is a transcription factor, was localized in the nuclei of the differentiated cells.[51] Furthermore, the expression of collagen type X, a hypertrophic factor in the embryonic stage was considered as a big challenge during differentiation of BM-MSCs and ADSCs to chondrocytes.[52] In this study, the expression of type X collagen gradually decreased compared with control, which is associated with the increased production of type II collagen. Also, AGC can be seen as a positive sign to chondrogenic differentiation during 2 weeks.

CONCLUSIONS

In this study, we were able to demonstrate the brilliant effect of three-dimensional scaffold consistent of CNTs and alginate hydro-gel, in improvement of gene expression related to chondrogenesis, which includes not only increased

expression of essential cartilage markers, but also decreased expression of negative marker in this process. Therefore, CNTs provided a supportive matrix for cartilage precursor cells that could be used to promote chondrogenic differentiation for cartilage TE applications in the future.

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