Thymoquinone loading into hydroxyapatite/alginate scaffolds accelerated the osteogenic differentiation of the mesenchymal stem cells

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Research

Keywords: Thymoquinone, Hydroxyapatite, Alginate, Scaffold, Mesenchymal stem cell

Posted Date: May 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-467670/v1>

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Version of Record: A version of this preprint was published at BioMedical Engineering OnLine on August 4th, 2021. See the published version at <https://doi.org/10.1186/s12938-021-00916-1>.

Abstract

Background: Hydroxyapatite (HA) can be loaded by some osteogenic inducing agents such as thymoquinone (TQ) and alginate. This study was performed to investigate the effect of TQ loading into HA/alginate scaffolds on osteogenic differentiation capability of mesenchymal stem cells.

Methods: HA scaffolds were fabricated by casting and sintering method and impregnated by TQ containing alginate. The stem cells were loaded onto the scaffolds and induced to differentiate into osteoblasts. Alkaline Phosphatase (ALP) activity, Alizarin Red S, Real-Time qRT-PCR, and MTT assessments were done. Finally, the cells were examined with a light microscope, confocal microscope, and SEM.

Results: The results showed that the presence of the alginate decelerates the degradation rate and reinforces the mechanical strength. while the presence of TQ had no significant influence on physical and mechanical properties of the HA/alginate scaffolds, it led to a significant increase in ALP activity and expression of collagen, osteopontin, and osteocalcin at early phase of differentiation. Also, TQ administration had no impact on calcium deposition and proliferation as well as bone-marker expression at long term differentiation.

Conclusion: TQ accelerates the differentiation of the stem cells into the osteoblasts without changing the properties of the scaffolds, and the HA/alginate/TQ scaffold can be used as a scaffold with osteogenic properties in bone tissue engineering applications.

Background

Repair of bone fractures due to trauma or degenerative diseases has become one of the most important concerns of regenerative medicine nowadays [1]. Although autografts and allografts are considered to be the best methods for repairing bone defects, complications limit these therapeutic approaches; it has also promoted the development of alternative technologies to solve these problems [2, 3]. Bone tissue engineering, as a more advanced alternative, involves delivering the cells to the area of injury, mimicking the normal bone tissue [4]. The main goal of tissue engineering is to create a suitable synthetic or natural alternative for the graft, which mainly consists of the scaffold, living cells, and growth factors [5]. One of the most widely used composites in bone scaffolds is hydroxyapatite(HA) with excellent osteoconductive and osteoinductive properties [6].

HA ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is the most stable ceramic compound of calcium phosphate under physiological conditions [7]. Fifty percent of the volume, 70% of the weight, and about 60% of the mineral fraction of human dry bone have a modified form of HA [8, 9]. Nowadays, due to its outstanding properties such as biocompatibility, bioactivity, osteoconductivity, and non-toxic nature, HA has many applications in tissue engineering [10, 11]. The results of research on HA show its effective and useful role in the process of bone regeneration and repair. Studies have shown that HA-hyaluronic acid-calcium sulfate scaffold improves the repair of the defects in the alveolar bone [12]. Xin Qi et al. showed that the use of a three-

dimensional (3D) HA-collagen-polycaprolactone scaffold increases the bone repair in the rat model [13]. HA can be loaded by some osteogenic inducing agents such as thymoquinone (TQ) to accelerate the bone-building process.

TQ (2-Isopropyl-5-methylbenzo-1,4-quinone) is the bioactive substance of *Nigella sativa* oil [14]. It has anti-fungal, anti-tumor, liver protective, anti-inflammatory, anti-epileptic, anti-coagulant, anti-diabetic, anti-hypertensive, and anti-bacterial effects [14–18]. TQ has been widely studied, and it has been also detected as an effective compound on the bone health [19, 20]. Several mechanisms have supported the potential effects of TQ on improving bone diseases such as rheumatoid arthritis [21, 22]. TQ accelerates bone formation by inducing Bone morphogenic protein 2 (BMP-2) through ERK signaling pathway [23, 24]. Wirries et al. (2013) showed that TQ induced osteoblast progenitor cell proliferation (MC3T3-E1), osteoblast differentiation, and mineral matrix deposition [24].

As the drug release from HA scaffolds is rapid, to delay and regulate it, a series of materials such as alginate is suggested to be used. Alginate is an available, cheap, and biocompatible biopolymer that is effective in bone tissue repair as well and has a wide range of applications in bone tissue engineering [25]. Studies showed that alginate in combination with HA and chitosan can be used as a suitable drug delivery system to regulate the rate of drug release in bone tissue engineering [26]. Another study showed that the combination of alginate with HA-collagen scaffold could mimic a suitable substrate with high biocompatibility for osteoblasts [27]. It has also been shown that porous calcium-alginate scaffolds can support bone differentiation and osteoblast growth and proliferation [28]. Considering the positive effects of HA, alginate and TQ on bone, in the current study, we prepared a 3D HA/alginate scaffold and the effect of the presence TQ with and without osteogenic medium (OM) in the osteogenic differentiation of human adipose-derived mesenchymal stem cells (hADMSCs) towards bone cells as well as the activity of differentiated cells was evaluated.

Result

Evaluation of purity and size of extracted HA particles

As shown in Fig. 1, the peaks in the XRD spectra of the extracted mineral components correspond to those of commercial HA. This indicates that most of the extracted mineral components are HA with high purity (Fig. 1-A, B). Also, the particle size of the extracted powder was estimated as 409.78 ± 89.58 nm (Fig. 1-C).

Scaffolding features

The porous scaffolds were made at a size of $10 \times 10 \times 3$ mm³ and the porosity was estimated as $72.75 \pm 3.375\%$. The data indicated that the trend of degradation rate was constant in all scaffolds regardless of loading TQ. The presence of the alginate decelerates the degradation rate significantly compared to the

control scaffolds after day 3 ($P < 0.05$). The presence of TQ had no significant influence on degradation rate of the scaffolds (Fig. 2-A).

Hydration test revealed that all the scaffolds absorbed water at full capacity almost immediately after immersion. The presence or absence of TQ did not show any significant impact on the hydration rate (Fig. 2-B).

Mechanical strength test showed that the presence of alginate, but not TQ, reinforced the mechanical strength compared to the control scaffolds ($P = 0.01$ for TO containing, and $P = 0.02$ for scaffold without TQ). The mechanical strength of the control scaffold was 3.28 ± 0.76 MPa, while that of alginate containing scaffolds with or without TQ was 5.20 ± 0.48 MPa and 4.72 ± 0.54 MPa, respectively. (Fig. 2-C). The result obtained from these tests revealed that the presence of TQ had no detrimental influence on physical properties of the scaffolds.

Characterization of hADMSC

The isolated cells were initially spindle-shaped and short, but gradually its phenotype became fibroblast-like. Flow cytometry data showed that the cells isolated from the adipose tissue expressed surface mesenchymal markers including CD44 (88.4%), CD105 (97.4%), CD106 (46.3%), while they failed to express the hematopoietic cell lineage marker, CD34 (2.10%), and endothelial cell marker, CD144, (0.78%) (Fig. 3).

Cytotoxicity assessment

A pilot study was conducted to find the appropriate dose of TQ for further investigations. MTT test showed that supplementation of 100 μ M TQ significantly reduced the cell viability compared with all the other groups ($P = 0.003$ for the 3rd day and $P < 0.0001$ for 7th day). The cell viability on the scaffolds loaded with two other doses, 50 and 25 μ M, was significantly similar to the control cultures, and they did not exert any cytotoxicity for hADMSCs. We choose the higher dose with lower cytotoxicity, 50 μ M, for further experiments. The number of viable cells in all the 3D groups was lower than the 2D groups ($P = 0.005$ for the third; $P < 0.0001$ for the fifth; and $P < 0.0001$ for the seventh days) (Fig. 4).

Measuring the rate of drug release

TQ release pattern showed that it sequestered during the first 1 min. The drug released at 20 min followed by a quick release at the first hour and then a constant release up to 6 hours. Then, the release of TQ accelerated with a higher rate up to 48 hours. (Fig. 5).

Cell adhesion test

The data from cell attachment test revealed that the presence of alginate had a significant positive effect on cell attachment, so that after 120 minutes, almost all the seeded cells were attached to the alginate

containing scaffolds ($P < 0.0001$). The presence of TQ had no detrimental effect on cell attachment (Fig. 6).

Mineralization assessments

ALP activity increased significantly in all the 3D cultures compared to 2D condition in the absence of OM, so that all the 3D culture systems provided a better condition for osteogenic differentiation of MSCs; however, the OM supplementation of 2D conventional culture condition led to a significant increase in ALP activity at late stage on days 21 and 28 ($P = 0.02$ and $P = 0.04$, respectively); all 3D conditions accelerated the osteogenesis, so that a significant higher ALP activity was observed compared to the corresponding 3D control cultures on days 7 and 14 ($P < 0.01$). The presence of TQ led to a significant increase in ALP activity compared to the matched control cultures at early phase of differentiation, on days 7 and 14 ($P < 0.0001$ for 3D + TQ + OM versus 3D-TQ + OM; $P = 0.04$ for 3D + TQ-OM versus 3D-TQ-OM on day 7; $P = 0.009$ for 3D + TQ + OM versus 3D-TQ + OM on day 14). This means that the TQ acted as an accelerator of osteoblast differentiation. At short term (7day), TQ reinforced the osteoinductive effect of 3D culture system since ALP activity showed a significant increase in the absence of OM and in the presence of TQ ($P = 0.04$). Besides, 3D HA scaffolds induced osteogenesis in MSC compared to 2D condition in the absence of OM ($P = 0.04$ on 7 day; $P < 0.0001$ on other days) (Fig. 7-A).

Alizarine Red S staining was used to evaluate calcium deposition by differentiated cells on the 28th day of differentiation. The results showed a significant more calcium deposition in the cultures supplemented by osteogenic medium compared to their counterpart without OM ($P < 0.001$). Also, the calcium deposition was higher in 3D cultures compared to the matched 2D conditions ($P < 0.0001$). TQ administration had no impact on calcium deposition. (Fig. 7-B).

Cell viability and proliferation assay after differentiation

After 28 day, the number of viable cells in all the groups which received OM was significantly fewer compared to the corresponding control cultures ($P < 0.0001$ for 3D + TQ + OM versus 3D + TQ-OM; $P = 0.0006$ for 3D-TQ + OM versus 3D-TQ-OM and $P < 0.0001$ for 2D + OM versus 2D-OM). Cell proliferation on both 2D conditions was higher compared to the matched 3D conditions ($P < 0.0001$ for all). TQ supplementation had no significant effect on cell proliferation ($P > 0.05$) (Fig. 7-C).

Real time RT-PCR

The expression of collagen 1, osteopontin, and osteocalcin genes significantly increased as the time progressed ($P < 0.001$). On both days 7 and 28, adding OM led to a significant increase in all the osteogenic marker expressions ($P < 0.05$).

On day 7, regardless of receiving osteogenic medium, the cells in the cultures supplemented with TQ expressed a significantly higher level of collagen ($P = 0.02$ for cultures treated with OM, $P = 0.01$ for cultures without OM) and osteocalcin ($P = 0.02$ for cultures treated with OM; and $P = 0.04$ for cultures

without OM) compared to the matched controls, in osteogenic medium-treated cultures, the expression of osteopontin increased significantly by TQ administration ($P = 0.03$).

On day 28, TQ administration did not exert any significant impact on the bone-specific marker expression. Thus, TQ acted as an accelerator of the induction of differentiation that led to early expression of bone-specific markers. Also, the cells cultured on HA scaffolds could express bone-specific markers, even in conditions without OM and TQ, and the level of expression increased as the time progressed. (Fig. 8).

Optical microscope

After osteogenesis differentiation, the morphology of the cells and their distribution were examined by paraffin-embedded sections and H&E staining. Most of the cells embedded within alginate showed spherical phenotype without prominence processes. However, some of them contained elongated nuclei. They showed Heterogeneous distribution throughout the scaffold. There was no significant difference in the cell morphology of the cells cultured in various conditions (Fig. 9).

Confocal Raman microscope

Generally, spectroscopy in Raman shifts of $500\text{--}1000\text{ cm}^{-1}$ was more related to the bonds present in the mineral and those of $1000\text{--}2000\text{ cm}^{-1}$ were related to the bonds of the organic part of the scaffold. Peaks of the Raman spectra represent a number of bonds in one or several special substances; Raman peaks at 589 cm^{-1} , 740 cm^{-1} , 955 cm^{-1} and 962 cm^{-1} and 1044 cm^{-1} were assigned for the vibration of $\nu_4\text{ po}_4^{3-}$ symmetric bonds (related to HA phosphate) [29], deformed bonds of nitro groups[30], for the vibration of calcium phosphate[31], administration of the vibration of symmetrical bonds related to calcium HA and HA mineral phosphate, respectively, that indicate the presence of HA as the primary base of the scaffolds. Raman peaks at 1076 cm^{-1} , 1124 cm^{-1} [29] and 1405 cm^{-1} [32] are related to lipids in the cell membranes, and 1615 cm^{-1} are related to the amino acids tyrosine, tryptophan and adenine, $1640\text{--}1680\text{ cm}^{-1}$ for Amide I [29] which indicated the presence of protein production such as collagen by the cells in the cultures. Also, the vibration at 1721 cm^{-1} [33], 1802 cm^{-1} , and 1869 cm^{-1} [34] represented CO binding, which may indicate the presence of protein or alginate. Also, the Raman shift of 1900 cm^{-1} is related to the $\text{C}=\text{C}$ bond of benzenoid rings [35]. Overall, Raman spectra revealed a shift to right for peak at 962 to 955 cm^{-1} . These peaks belong to minerals[29, 31]. Also, the trend of Raman spectra in the cultures which received just TQ was the same as both cultures which received OM, while the trend of the cultures deprived of either TQ or OM differed from the others. This indicates that the TQ may have an osteoinductive property (Fig. 10).

Scanning electron microscopy

SEM images showed that alginate filled the pores of the HA scaffolds. The mean value of the pore size of the HA scaffold was $411.32 \pm 32.67\text{ }\mu\text{m}$. The phenotype of the cells in the cultures which received TQ with or without OM and the cultures which received OM was round with a few short processes which was

similar to osteoblasts. The cells in the cultures deprived of OM and TQ expanded well with some processes. These cells had the typical phenotype of MSCs. (Fig. 11).

Discussion

The current evidence supports the impact of TQ-based scaffold on elevating the osteoblast differentiation capacity of MSCs without considerable impact on the physical and mechanical properties of the scaffold. Quinones with a wide range of biological activities and applications have several beneficial effects on bone formation. In this regard, both animal and human-based studies could demonstrate its preventive and even therapeutic effects on osteoporotic condition [36, 37]. From a physiological point of view, quinones can inhibit the bone resorption, reduce the osteoclastic activation, as well as accelerate proliferation and differentiation of the osteoblasts, leading to skeletal recovery [38, 39]. TQ, as a compound derived from *Nigella sativa* L. and one of the most widely used compounds, has anti-inflammatory, anti-oxidant, anti-cancer, analgesic, and even hypoglycemic properties [40–42]. Recently, much attention has been paid to its beneficial effects on bone formation and recovery of bone injuries via impact of bone metabolism. Our study showed the effectiveness of loading the TQ on HA/Alginate scaffolds to accelerate osteoblast differentiation and this is helpful in bone tissue engineering applications. The data from the current study showed that the bone specific markers such as osteopontin, osteocalcin, collagen type I expressed as soon as the 7th day compared to the cells cultured in TQ-free conditions. ALP activity test also confirmed this finding. In vivo studies in animal models have indicated the beneficial effects of TQ on bone formation. TQ has been reported to accelerate in vivo new bone formation by increasing the number of osteoblasts and also inhibiting the inflammatory pathways in rats [41]. Anti-inflammatory impact of TQ leads to inhibition of osteoclastogenic [23], and osteoclasts induce hyperactivation of the osteoblasts and increase in bone formation [43]. It has been previously reported that supplementation of culture media with TQ induced the expression levels of alkaline phosphatase, osteocalcin, and osteopontin, but not collagen type I [24] in the osteoblast cell line through the upregulation of bone morphogenic protein. The results of the current study indicated that TQ accelerated the osteoblast differentiation as well. We also found that TQ could boost the expression level of collagen type I as well as the other bone-markers, of course when administrated during osteoblast differentiation.

Both HA and alginate have been shown to exert osteogenic impact on MSCs [44, 45]. The results showed that TQ not only accelerated the osteoblast differentiation in the presence of osteogenic medium, but also elevated the osteoinductive property of HA scaffold on hADMSCs. To find whether TQ can induce osteogenic differentiation in the absence of the other osteogenic stimulators needs more investigations. The findings of the current study showed that TQ loading either accelerated or enhanced the osteoblast differentiation since at long term, the cells cultured on all the TQ-loaded and TQ-free scaffolds expressed statistically similar bone-specific markers or showed the same ALP activity and mineralization. This may be due to the cells both matched conditions acquired the final osteogenic phenotype at long time. On the other hand, although alginate decelerated TQ releasing rate, its concentration was too low to enhance the osteogenesis at days 21st and 28th. However, ALP activity showed that 3D culture system induced

osteogenesis in all conditions. This data confirmed the previous study that reported 3D HA scaffolds had osteoinductive property [44]. Besides osteogenic effects, alginate has been used for drug delivery as well. TQ encapsulation in polylactide-co-glycolide acid nanoparticles can enhance its biological properties such as anti-proliferative or anti-inflammation activities [46]. Also, the anti-oxidant feature of TQ loaded in poly (sodium N-undecylenyl-valinate) was higher than free TQ [47]. TQ is an unstable molecule in aqueous solutions [48]. Encapsulation in alginate has been reported to enhance the TQ stability [49]. Alginate has been suggested as a good biometrical for improving the stability of active molecules [48]. In this study, TQ entrapped in alginate and drug release test showed that the release of TQ decelerated without any significant influence on physical and mechanical properties of the scaffold, and after 48 hours, TQ was yet released into the culture medium. The effect of TQ on viability, proliferation, and immunomodulatory potential of bone marrow-derived mesenchymal stem cells (BM-MSCs) was assessed in vitro. TQ reduced the cell viability in a time- and dose-dependent manner [50]. The dose and time dependent cytotoxicity of TQ was also detected in some cancer cell lines [51, 52]. Our data also confirm the cytotoxic effects of TQ in higher doses, but it was not time-dependent. We checked the osteogenic induction of the highest non-toxic dose of TQ.

Conclusion

Generally, TQ accelerates the differentiation of stem cells into the osteoblast with no change in the physical and mechanical properties of the scaffolds, and the HA/ alginate /TQ scaffold can be used as a scaffold with osteogenic properties in bone tissue engineering applications.

Methods

Extraction of HA and characterization

First, the diaphysis of the cow's femur was boiled for 2–3 hours followed by washing in water to remove the excess tissue, cut into small pieces, delipidized in acetone, and dried overnight at 100°C. Extraction of HA was done by thermal decomposition method and using electric furnace (LAC, Ltd, Czech Republic) at 850°C for 3 hours; the powder obtained was pulverized by Planetary Ball Mill (Mpm-191, Iran). The X-ray diffraction method (XRD, Bruker, USA) was used to evaluate the purity of the extracted powder by comparing it with commercially purchased HA powder (Merck). CuK α beam was used for analysis. The scanning rate was equal to 1.54 degrees per minute and the diffraction angle was from 10 to 90 degrees. Also, to measure the particle size, we used SEM analysis of the extracted powder alongside Image J software (<http://imagej.nih.gov/ij/index.html>).

Scaffold construction

First, 6g of HA powder was added to 14 mL of distilled water and mixed on a stirrer for 5 min. An equivalent of 7% of HA powder (0.42 g) of triethyl phosphate (TEP; (C₂H₅)₃PO₄) (Sigma-Aldrich, USA) was added to the mixture and slurry was mixed on the stirrer for 24 hours. Then, an equivalent of 7% of HA

powder (0.42 g), of glycerin (Sigma-Aldrich, USA) was added and stirred for 1.5 hours. Polyvinyl alcohol (PVA) (Sigma-Aldrich, USA) was prepared at a concentration of 15% and an equivalent of 33% of the weight of HA powder (2 g) was added to the slurry and mixed for 24 hours. Polyurethane foam was used as a template and cut to the desired size and shape. They were immersed in slurry and dried at 90°C for 15 minutes. The immersing and drying periods of the foams were repeated twice, and finally the slurry-coated foams were heated at a rate of 2°C/min to reach the temperature of 800°C; then, they remained at a temperature of 800°C for 5 hours. After that, the temperature increased at a speed of 3.5°C/min to reach the temperature of 1250°C and led them to sinterized for 3 hours. The temperature of the device was then dropped to ambient temperature.

The scaffolds were immersed in a 1% alginate solution (Sigma-Aldrich, USA) dissolved in phosphate-buffered saline (PBS) with/without TQ (Sigma-Aldrich, USA). To avoid trapping air bubbles, we placed them inside the vacuum pump machine for 10 minutes. Then, the gelation was done by adding 1% calcium chloride solution (Merck, Germany).

Characterization of the scaffold

SEM images were prepared by lyophilizing TQ free scaffolds. Gold replica was prepared using Q150R- ES sputter coater (Quorum Technologies, London, UK) and imaged using an VEGA3 microscope (TESCAN, Brno, Czech Republic) at 10 kV accelerating voltage. The pore size was estimated by analyzing SEM images using image J software. The porosity was measured by liquid exchange method.

The degradation rate was measured by incubating the scaffolds in 0.01% trypsin (Bioidea, Iran) and weighting them on days 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, and 56.

The hydration rate was evaluated by immersing lyophilized TQ loaded scaffolds in distilled water and weighting them at the beginning as well as at intervals of 5, 15, 30, 60, 120, and 240 seconds.

For evaluating the impact of TQ loading on mechanical strength, the scaffolds at a size of 10×10×3 mm³ and the same porosity were exposed to a mechanical resistance determination device (Zwick/Roell, Germany) at a speed of 0.5 mm/min.

TQ releasing assessment

To evaluate the drug release, we prepared the scaffolds according to what was mentioned in the previous part. Then, 1 mL of distilled water was added to each scaffold and the supernatant was removed at 20, 40, 60 seconds, 20, 40, 60 minutes, and 2, 4, 6, 12, 24, 48 hours. Finally, the release of TQ was assessed using the High-Performance Liquid Chromatography (HPLC AZURA; KNAUER, Germany) with a C18 column. The mobile phase consisted of a solvent system of acetonitrile and water with a ratio of 45:55. The flow rate was performed at 1 mL/min at 30°C and detection wavelength was at 254 nm.

Isolation, culture, and characterization of hADMSC

Adipose tissue samples from female patients undergoing liposuction under the age of 27–29 years were transferred to the laboratory under sterile conditions. The adipose tissue was cut into small pieces and treated with 0.2% collagenase type I (Gibco) in the serum-free medium for 45 minutes; then, the suspension was centrifuged for 5 minutes at 1500 rpm and the cell pellets were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing L-glutamine (1mM), Pen-Strep (1%) and 20% fetal bovine serum (FBS) (Gibco) and incubated at 37°C and 5% CO₂. After 48 hours, the float cells were discarded, and the culture media was changed.

To characterize the hADMSCs, the cells were exposed to Fluorescein-5-isothiocyanate (FITC)-conjugated CD144 and CD44, to Phycoerythrin (PE)-conjugated CD34 and CD106, and to Peridinin-Chlorophyll-protein (PerCP)-conjugated CD105 antibodies all from Abcam. Finally, the percentage of expression of these CD markers was determined by flow cytometry using Flow Cytometer laser 488 nm (Becton Dickinson, NJ, USA), and Flowjo software was employed for analysis.

Differentiation of ADMSCs

The cells were aliquoted into 6 groups. The aliquotes at a density of 1×10^6 cells per mL were mixed with alginate with or without TQ and OM and seeded into the HA scaffolds and gelation induced by adding CaCl₂. Two of them were cultured on a 2D thin alginate coat with or without OM for 7 and 28 days. OM included 0.1 mM dexamethasone, 50 µM ascorbic acid, and 10 mM beta-glycerophosphate (Sigma-Aldrich) in DMEM medium. The culture media with or without OM contained 10% FBS (Gibco). The medium was changed every three days.

Cytotoxicity assessment

The 3-(4,5 dimethyl-2-thiazolyl) -2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich, USA) was performed at 3, 5, and 7 days after cell seeding to determine the appropriate and non-toxic dose of TQ and cell viability on the scaffolds. Briefly, to do this, TQ was prepared in 1% alginate at concentrations of 25, 50, and 100 µM. Then, 3×10^4 MSC/well was added to the alginate; they were impregnated into the HA scaffolds and allowed to be jellified by adding CaCl₂. After 3, 5, and 7 days, the culture media were replaced with 500 µL of 0.5 mg/mL MTT and incubated at 37°C for 3 hours. The MTT was then eluted from the vital cells by adding 300 µL of dimethyl sulfoxide (DMSO; Merck) and incubating for 30 min. The optical density (OD) of eluted MTT was measured using a POLARstar Omega Plate Reader Spectrophotometer at 595 nm.

Cell differentiation assessments

To be sure about the presence of viable cells after a 28-day period of differentiation, we performed another MTT assay as described previously. The cell phenotype on the scaffolds after differentiation was obtained by SEM image.

Alkaline Phosphatase Activity

The alkaline phosphatase (ALP) activity was evaluated on days 7, 14, 21, and 28 of differentiation using a commercial kit (Pars Azmun, Iran) as a functional test for the osteoblasts. The basis of this kit is the conversion of colorless nitrophenyl phosphate to yellow nitrophenol and it was done according to the manufacturer's instructions. The yellow intensity of nitrophenol was measured with a plate reader spectrophotometer at 405 nm.

Alizarin Red S staining

Alizarin red S staining was used to evaluate the amount of calcium deposition by the osteoblast cells. After 28 days of differentiation, 2D and 3D cultures were fixed with 4% PFA for 20 min. The samples were exposed to a 1% Alizarin red S solution (Sigma-Aldrich) for 30 minutes. The sedimented dye was then eluted with 500 µl of 100 mM Cetyl pyridinium chloride monohydrate (Merck) and its optical density was measured with a plate reader spectrophotometer at 405 nm.

Cell attachment test

First, 3×10^4 cells were mixed with 1% alginate with/without TQ and seeded on the scaffolds. Then, 1% calcium chloride solution was added on them to start gelation of alginate and they were incubated in a serum-free environment for 2 hours. The scaffolds were then placed vertically in a falcon tube and centrifuged at 30 rpm for 30 minutes. The number of detached cells, as pellet, were then counted using a Neubauer chamber and subtracted by the number of initial cell number.

Real-Time qRT-PCR

Total RNA from differentiated osteoblast was isolated using the TRIzol (YTzol pure RNA, CinnaGen, Tehran, Iran), according to the company's instructions. Extracted RNA content was quantified by NanoPhotometer (NanoDrop 2000, Implen, Germany). cDNA was produced with the aid of a High-Capacity RNA to cDNA kit (Thermo Fisher Scientific, USA), according to the manufacturer's protocol. The expression of Collagen I, Osteocalcin and Osteopontin was evaluated by qRT-PCR (ABI7900HT, USA). Specific primer sequences, as depicted in Table 1. PCR conditions were as follows: 10 min of initial denaturation at 94°C, followed by 40 cycles of denaturation at 95°C for 15 s; annealing was conducted at 60°C for 45 s, followed by 30 s extension at 72°C, and 5 min final extension at 72°C. PCR results were quantitatively analyzed by Rotor-Gene Q Series Software. For data analysis, the fold change (FC) of each gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method, whereas housekeeping genes, β -actin and GAPDH, were used as controls to normalize the results.

Confocal Raman microscope

After 28 days of differentiation, all the 3D samples were fixed by 4% PFA for 20 min and then they were lyophilized; the samples were evaluated by Lab-Ram HR Confocal Raman spectrometer (Horiba, Japan). The spectra were prepared with an excitation wavelength of 633 nm and a maximum power of 17 mW from different parts of the scaffold, including filled pores with alginate, HA blades, and cells. To include organic and mineral excitations, Raman shift was investigated at the range of 500–2000 cm^{-1} .

Optical microscope

After 28 days of differentiation, the 3D groups were fixed using 20% PFA for 20 minutes and prepared for paraffin-embedded light microscopy. Due to the presence of HA in the samples and in the absence of demineralization during tissue preparation, the blocks were placed in 8% HCl and 8% formic acid from the starting of tissue cut point, to prevent the scaffolding from pulverizing during cutting. The samples were sectioned at 20 μ m thickness, and stained with hematoxylin and eosin (H&E) and examined by a light microscope (Nikon E-200 microscope, Japan) and imaged.

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). GraphPad Prism software (version 8.1) was used to quantitatively analyze the data and draw the graphs, and the data were analyzed using one way-ANOVA and two way-ANOVA statistical tests. P-value with less than 0.05 was considered as significant data. All experiments were performed in triplicate.

Declarations

Acknowledgments

The project was assigned in the office of the Vice-Chancellor of Research Affairs, Shiraz University of Medical Sciences. The authors would like to thank Shiraz University of Medical Sciences, Shiraz, Iran and also Center for Development of Clinical Research of Nemazee Hospital and Dr. Nasrin Shokrpour for editorial assistance.

Authors' contributions

ERM performed the experiments and was involved in the collection, analysis, and interpretation of data and manuscript drafting. TTK and ZV conceived the original idea, supervised the project and interpreted the data. ERM, TTK and ZV Critical revision of article. VZ assisted in data collection. ZV involved in found acquisition. All authors read and approved the final manuscript.

Funding

This study was supported by the Vice-Chancellor of Research in Shiraz University of Medical Sciences by offering a grant no. 98-01-01-19725.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shiraz University of Medical Sciences (Registration number: IR.SUMS.REC.1398.718).

Consent for publication

No applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

ALP: Alkaline Phosphates;

BMPs: Bone Morphogenetic Proteins;

DMEM: Dulbecco's Modified Eagle's Medium;

FBS: Fetal Bovine Serum;

HA: Hydroxyapatite;

H&E: Hematoxylin & Eosin;

HPLC: High-Performance Liquid Chromatography;

hADMSCs: human Adipose-Derived Mesenchymal Stem Cells;

MSC: Mesenchymal Stem Cell;

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

OM: Osteogenic Medium;

OD: Optical Density;

PBS: Phosphate Buffer Saline;

PFA: Paraformaldehyde;

PVA: Polyvinyl Alcohol;

SEM: Scanning Electron Microscopy;

TQ: Thymoquinone;

TEP: Triethyl Phosphate

XRD: X-ray Power Diffraction.

References

1. Tang D, Tare RS, Yang L-Y, Williams DF, Ou K-L, Oreffo RO. Biofabrication of bone tissue: approaches, challenges and translation for bone regeneration. *Biomaterials*. 2016;83:363–82.
2. Gupta P, Adhikary M, Kumar M, Bhardwaj N, Mandal BB. Biomimetic, osteoconductive non-mulberry silk fiber reinforced tricomposite scaffolds for bone tissue engineering. *ACS Appl Mater Interfaces*. 2016;8(45):30797–810.
3. Grémare A, Guduric V, Bareille R, Heroguez V, Latour S, L'heureux N, et al. Characterization of printed PLA scaffolds for bone tissue engineering. *Journal of Biomedical Materials Research Part A*. 2018;106(4):887–94.
4. Fu Q. Bioactive Glass Scaffolds for Bone Tissue Engineering. *Biomedical, Therapeutic and Clinical Applications of Bioactive Glasses*. Elsevier; 2019. pp. 417–42.
5. Chen L, Li B, Xiao X, Meng Q, Li W, Yu Q, et al. Preparation and evaluation of an Arg-Gly-Asp-modified chitosan/hydroxyapatite scaffold for application in bone tissue engineering. *Mol Med Rep*. 2015;12(5):7263–70.
6. Huang Y, He J, Gan L, Liu X, Wu Y, Wu F, et al. Osteoconductivity and osteoinductivity of porous hydroxyapatite coatings deposited by liquid precursor plasma spraying: in vivo biological response study. *Biomed Mater*. 2014;9(6):065007.
7. Chen S, Du X, Wang T, Jia L, Huang D, Chen W. Synthesis of near-infrared responsive gold nanorod-doped gelatin/hydroxyapatite composite microspheres with controlled photo-thermal property. *Ceram Int*. 2018;44(1):900–4.
8. Kadu K, Ghosh G, Panicker L, Kowshik M, Ramanan SR. Role of surface charges on interaction of rod-shaped magnetic hydroxyapatite nanoparticles with protein. *Colloids Surf B*. 2019;177:362–9.
9. Gayathri B, Muthukumarasamy N, Velauthapillai D, Santhosh S. Magnesium incorporated hydroxyapatite nanoparticles: preparation, characterization, antibacterial and larvicidal activity. *Arabian journal of chemistry*. 2018;11(5):645–54.
10. Degli Esposti M, Chiellini F, Bondioli F, Morselli D, Fabbri P. Highly porous PHB-based bioactive scaffolds for bone tissue engineering by in situ synthesis of hydroxyapatite. *Materials Science Engineering: C*. 2019;100:286–96.
11. Lin W-C, Yao C, Huang T-Y, Cheng S-J, Tang C-M. Long-term in vitro degradation behavior and biocompatibility of polycaprolactone/cobalt-substituted hydroxyapatite composite for bone tissue engineering. *Dent Mater*. 2019;35(5):751–62.
12. Subramaniam S, Fang Y-H, Sivasubramanian S, Lin F-H, Lin C-p. Hydroxyapatite-calcium sulfate-hyaluronic acid composite encapsulated with collagenase as bone substitute for alveolar bone regeneration. *Biomaterials*. 2016;74:99–108.
13. Qi X, Huang Y, Han D, Zhang J, Cao J, Jin X, et al. Three-dimensional poly (ϵ -caprolactone)/hydroxyapatite/collagen scaffolds incorporating bone marrow mesenchymal stem cells for the repair of bone defects. *Biomedical materials*. 2016;11(2):025005.

14. Armutcu F, Akyol S, Akyol O. The interaction of glutathione and thymoquinone and their antioxidant properties. *Electronic Journal of General Medicine*. 2018;15(4).
15. Khader M, Eckl PM. Thymoquinone: an emerging natural drug with a wide range of medical applications. *Iranian journal of basic medical sciences*. 2014;17(12):950.
16. Salahshoor MR, Haghjoo M, Roshankhah S, Makalani F, Jalili C. Effect of thymoquinone on reproductive parameter in morphine-treated male mice. *Advanced biomedical research*. 2018;7.
17. Badr G, Alwasel S, Ebaid H, Mohany M, Alhazza I. Perinatal supplementation with thymoquinone improves diabetic complications and T cell immune responses in rat offspring. *Cellular immunology*. 2011;267(2):133–40.
18. Kanter M. Protective effects of quercetine on the neuronal injury in frontal cortex after chronic toluene exposure. *Toxicol Ind Health*. 2013;29(7):643–51.
19. Bao L, Qin L, Liu L, Wu Y, Han T, Xue L, et al. Anthraquinone compounds from *Morinda officinalis* inhibit osteoclastic bone resorption in vitro. *Chem Biol Interact*. 2011;194(2–3):97–105.
20. Al-Amri A, Bamosa A. Phase I safety and clinical activity study of thymoquinone in patients with advanced refractory malignant disease. *Shiraz E-Med J*. 2009;10:107–11.
21. Vaillancourt F, Silva P, Shi Q, Fahmi H, Fernandes JC, Benderdour M. Elucidation of molecular mechanisms underlying the protective effects of thymoquinone against rheumatoid arthritis. *Journal of cellular biochemistry*. 2011;112(1):107–17.
22. Umar S, Zargan J, Umar K, Ahmad S, Katiyar CK, Khan HA. Modulation of the oxidative stress and inflammatory cytokine response by thymoquinone in the collagen induced arthritis in Wistar rats. *Chemico-Biol Interact*. 2012;197(1):40–6.
23. Thummuri D, Jeengar MK, Shrivastava S, Nemani H, Ramavat RN, Chaudhari P, et al. Thymoquinone prevents RANKL-induced osteoclastogenesis activation and osteolysis in an in vivo model of inflammation by suppressing NF-KB and MAPK Signalling. *Pharmacol Res*. 2015;99:63–73.
24. Wirries A, Schubert A-K, Zimmermann R, Jabari S, Ruchholtz S, El-Najjar N. Thymoquinone accelerates osteoblast differentiation and activates bone morphogenetic protein-2 and ERK pathway. *Int Immunopharmacol*. 2013;15(2):381–6.
25. Lee KY, Mooney DJ. Alginate: properties and biomedical applications. *Prog Polym Sci*. 2012;37(1):106–26.
26. Supramaniam J, Adnan R, Kaus NHM, Bushra R. Magnetic nanocellulose alginate hydrogel beads as potential drug delivery system. *Int J Biol Macromol*. 2018;118:640–8.
27. Zhang S, Cui F, Liao S, Zhu Y, Han L. Synthesis and biocompatibility of porous nano-hydroxyapatite/collagen/alginate composite. *Journal of Materials Science: Materials in Medicine*. 2003;14(7):641–5.
28. Chen C-Y, Ke C-J, Yen K-C, Hsieh H-C, Sun J-S, Lin F-H. 3D porous calcium-alginate scaffolds cell culture system improved human osteoblast cell clusters for cell therapy. *Theranostics*. 2015;5(6):643.

29. Talari ACS, Movasaghi Z, Rehman S, Rehman IU. Raman spectroscopy of biological tissues. *Appl Spectrosc Rev.* 2015;50(1):46–111.
30. Raj A, Raju K, Varghese HT, Granadeiro CM, Nogueira HI, Panicker CY. IR, Raman and SERS spectra of 2-(methoxycarbonylmethylsulfanyl)-3, 5-dinitrobenzene carboxylic acid. *J Braz Chem Soc.* 2009;20(3):549–59.
31. Stammeier JA, Purgstaller B, Hippler D, Mavromatis V, Dietzel M. In-situ Raman spectroscopy of amorphous calcium phosphate to crystalline hydroxyapatite transformation. *MethodsX.* 2018;5:1241–50.
32. Beattie JR, Brockbank S, McGarvey JJ, Curry WJ. Raman microscopy of porcine inner retinal layers from the area centralis. *Mol Vis.* 2007;13:1106.
33. Ditta A, Nawaz H, Mahmood T, Majeed M, Tahir M, Rashid N, et al. Principal components analysis of Raman spectral data for screening of Hepatitis C infection. *Spectrochim Acta Part A Mol Biomol Spectrosc.* 2019;221:117173.
34. Lu H-C, Ghosh S, Katyal N, Lakhanpal VS, Gearba-Dolocan IR, Henkelman G, et al. Synthesis and Dual-Mode Electrochromism of Anisotropic Monoclinic Nb₁₂O₂₉ Colloidal Nanoplatelets. *ACS Nano.* 2020;14(8):10068–82.
35. Ding S. Effects of tissue fixation on Raman spectroscopic characterization of retina. 2015.
36. El-Najjar N, Gali-Muhtasib H, Ketola RA, Vuorela P, Urtti A, Vuorela H. The chemical and biological activities of quinones: overview and implications in analytical detection. *Phytochem Rev.* 2011;10(3):353–70.
37. Sogabe N, Maruyama R, Baba O, Hosoi T, Goseki-Sone M. Effects of long-term vitamin K1 (phylloquinone) or vitamin K2 (menaquinone-4) supplementation on body composition and serum parameters in rats. *Bone.* 2011;48(5):1036–42.
38. Lee S-U, Shin HK, Min YK, Kim SH. Emodin accelerates osteoblast differentiation through phosphatidylinositol 3-kinase activation and bone morphogenetic protein-2 gene expression. *Int Immunopharmacol.* 2008;8(5):741–7.
39. Xiang M-X, Xu Z, Su H-W, Hu J, Yan Y-J. Emodin-8-O- β -D-glucoside from *Polygonum Amplexicaule* D. Don var. *Sinense* Forb. promotes proliferation and differentiation of osteoblastic MC3T3-E1 cells. *Molecules.* 2011;16(1):728–37.
40. Chen W-P, Tang J-L, Bao J-P, Wu L-D. Thymoquinone inhibits matrix metalloproteinase expression in rabbit chondrocytes and cartilage in experimental osteoarthritis. *Experimental Biology Medicine.* 2010;235(12):1425–31.
41. Kara MI, Erciyas K, Altan AB, Ozkut M, Ay S, Inan S. Thymoquinone accelerates new bone formation in the rapid maxillary expansion procedure. *Arch Oral Biol.* 2012;57(4):357–63.
42. Su J-L, Chiou J, Tang C-H, Zhao M, Tsai C-H, Chen P-S, et al. CYR61 regulates BMP-2-dependent osteoblast differentiation through the $\alpha\beta$ 3 integrin/integrin-linked kinase/ERK pathway. *J Biol Chem.* 2010;285(41):31325–36.

43. Chen X, Wang Z, Duan N, Zhu G, Schwarz EM, Xie C. Osteoblast–osteoclast interactions. *Connect Tissue Res.* 2018;59(2):99–107.
44. Latifi M, Talaei-Khozani T, Mehraban-Jahromi H, Sani M, Sadeghi-Atabadi M, Fazel-Anvari A, et al. Fabrication of platelet-rich plasma heparin sulfate/hydroxyapatite/zirconia scaffold. *Bioinspired Biomimetic Nanobiomaterials.* 2018;7(2):122–30.
45. Cai X, Lin Y, Ou G, Luo E, Man Y, Yuan Q, et al. Ectopic osteogenesis and chondrogenesis of bone marrow stromal stem cells in alginate system. *Cell Biol Int.* 2007;31(8):776–83.
46. Ravindran J, Nair HB, Sung B, Prasad S, Tekmal RR, Aggarwal BB. RETRACTED: Thymoquinone poly (lactide-co-glycolide) nanoparticles exhibit enhanced anti-proliferative, anti-inflammatory, and chemosensitization potential. *Elsevier*; 2010.
47. Ganea GM, Fakayode SO, Losso JN, Van Nostrum CF, Sabliov CM, Warner IM. Delivery of phytochemical thymoquinone using molecular micelle modified poly (D, L lactide-co-glycolide) (PLGA) nanoparticles. *Nanotechnology.* 2010;21(28):285104.
48. Alkhatib H, Mohamed F, Sabere ASBM, Akkawi ME, Doolaanea AA. Investigation of thymoquinone stability in black seed oil alginate beads. *Current Trends in Biotechnology & Pharmacy*; 2020.
49. Samak YO, Santhanes D, El-Massik MA, Coombes AG. Formulation strategies for achieving high delivery efficiency of thymoquinone-containing *Nigella sativa* extract to the colon based on oral alginate microcapsules for treatment of inflammatory bowel disease. *J Microencapsul.* 2019;36(2):204–14.
50. Alimoradi E, Sisakhtnezhad S, Akrami H. Thymoquinone influences the expression of genes involved in self-renewal and immunomodulatory potential of mouse bone marrow-derived mesenchymal stem cells in vitro. *Environmental toxicology pharmacology.* 2018;60:216–24.
51. Yazan LS, Ng WK, Al-Naqeeb G, Ismail M. Cytotoxicity of thymoquinone (TQ) from *Nigella sativa* towards human cervical carcinoma cells (HeLa). *Journal of Pharmacy Research.* 2009;2(4):585–9.
52. Alaofi OM, Noorwali A, Zahran F, Al-Abd AM, Al-Attas S. Cytotoxicity of thymoquinone alone or in combination with cisplatin (CDDP) against oral squamous cell carcinoma in vitro. *Scientific reports.* 2017;7(1):1–12.

Tables

Table 1. The promoter sequences used		
Name	Primer Sequence (5'→3')	Melting Point C°
Collagen 1	Forward: CGGCTCCTGCTCCTCTTAG Reveres: GGGCTCGGGTTTCCACACG	65
Osteocalcin	Forward: CCTCACACTCCTCGCCCTA Reveres: TCTTCACTACCTCGCTGCC	63
Osteopontin	Forward: CTCAGCCAAACGCCGACCAA Reveres: TCCTCAGAACTTCCAGAATCAGCCT	65
β-actin	Forward: GCCTTTGCCGATCCGC Reveres: GCCGTAGCCGTTGTCTG	55
GAPDH	Forward: GCAAGAGCACAAAGAGGAAGA Reveres: ACTGTGAGGAGGGGAGATTC	57

Figures

Figure 1

Evaluation of the purity of the extracted hydroxyapatite (HA); A. commercial HA XRD spectrum, B. XRD spectrum of extracted HA, XRD spectra showed that most of the mineral content is HA, C. SEM images of the HA particles. The particle size of the extracted powder was estimated to be 409.78 ± 89.58 nm.

Figure 2

Physical and mechanical properties of the scaffold; A. The degradation rate of scaffolds. In general, scaffolds with alginate with or without thymoquinone (TQ) had less corrosion than the control group, B.

Hydration rate of scaffolds. The amount of water absorption was similar in all the groups, C. Mechanical strength of the scaffolds by the diametral method. In general, the use of alginate increased the mechanical strength of the scaffolds compared to the control group. •significantly different between control and alginate-TQ groups and *significantly different between control and alginate+TQ groups in chart A. *significantly different from the controls in chart C. (*P<0.05, **P<0.01, ***P<0.001), (•P<0.05, ••P<0.01, •••P<0.001).

Figure 3

Flow cytometry showed that adipose-derived stem cells expressed CD44, CD105, and CD106.

Figure 4

Cell viability by MTT assay as a criterion for drug dose determination. In all groups, the viability rate of cells on the seventh day was higher than that of their counterparts on the third and fifth days. Also, the cell viability of the both 25 and 50 μ M of TQ-treated cultures was the same as matched controls. In all days, the cell viability in 2D condition was higher than all the 3D conditions. *significantly different from the controls on day 3 ($P < 0.01$), #significantly different from the controls on day 5 ($P < 0.001$), α significantly different from the controls on day 7 ($P < 0.001$).

Figure 5

The release of TQ from the scaffolds slowly continued into the environment up to 48 hours. Calibration curve for standard TQ (A), HPLC peak for a representative standard TQ (B), A representative sample in 60 seconds (C), and 24 hours (D). the releasing curve of TQ from scaffold during 48 hours (E).

Figure 6

The number of cells attached to the alginate-containing scaffolds with or without thymoquinone was significantly increased compared to the control group. *significantly different from the controls ($P < 0.0001$).

Figure 7

Cell differentiation assessments; A. Evaluation of alkaline phosphatase activity on different days of differentiation in 2D and 3D groups, B. calcium deposition by differentiated cells after 28 days, C. Evaluation of the cell viability at the end of differentiation period. Thymoquinone acts as an accelerator of the induction of differentiation in the early days. *significant difference between the groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 8

Expression of A. collagen I, B. osteopontin, C. osteocalcin genes in mesenchymal cells cultured on hydroxyapatite-alginate scaffolds in the presence and absence of osteogenic medium and thymoquinone. *significant difference between the groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (P=0.001 for 3D+TQ+OM versus 3D+TQ-OM, P=0.0008 for 3D-TQ+OM versus 3D-TQ-OM on 7 day; P<0.0001 for all groups with/ without OM on day 28 in collagen 1 expression), (P=0.003 for 3D+TQ+OM versus 3D+TQ-

OM, $P=0.03$ for 3D-TQ+OM versus 3D-TQ-OM on 7 day; $P<0.0001$ for 3D+TQ+OM versus 3D+TQ-OM, $P=0.0005$ for 3D-TQ+OM versus 3D-TQ-OM on day 28 in osteopontin expression), and ($P=0.005$ for 3D+TQ+OM versus 3D+TQ-OM, $P=0.01$ for 3D-TQ+OM versus 3D-TQ-OM on 7 day; $P<0.0001$ for all groups with/ without OM on day 28 in osteocalcin expression)

Figure 9

Light microscope images; A. 3D+TQ+OM, B. 3D+TQ-OM, C. 3D-TQ+OM, and D. 3D-TQ-OM groups. Generally, cells in the alginate were scattered non-uniformly. Also, any cell morphological changes were observed between the groups. (Arrowhead: cell, yellow arrow: hydroxyapatite, green arrow: alginate).

Figure 10

Raman confocal microscopy; A. Raman spectra related to mineral components at 500-1000 cm^{-1} , and B. Organic component at 1000-2000 cm^{-1} .

Figure 11

Scanning electron microscope images; A. Hydroxyapatite (HA) scaffold, B. HA scaffold containing alginate, C. Osteoblastic cells with short processes in the 3D+TQ+OM group, D. Cells with mesenchymal stem cell phenotype expanded on 3D-TQ -OM cultures.