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Original Article

Induced Chondrogenic Differentiation of hESCs by hESC-Derived MSCs Conditioned Medium and Sequential 3D-2D Culture System

Shayesteh Shahriary [1,2] M.Sc., Ehsan Farashahi-Yazd [1,3] Ph.D., Fatemeh Hajizadeh-Tafti [1] M.Sc., Fatemeh Akyash [1,3,4] Ph.D., Behrouz Aflatoonian [1,3,5,*] Ph.D.

[1] Stem Cell Biology Research Center, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[2] Department of Medical Biotechnology, Faculty of Medicine, Shahid Sadoughi University of. Medical Sciences, Yazd, Iran
[3] Department of Reproductive Biology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[4] Genetics and Infertility Unit, Erfan Hospital, Tehran, Iran
[5] Department of Advanced Medical Sciences and Technologies, Faculty of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

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ABSTRACT

Background and Aims: It has been proven that human mesenchymal stem cells (MSCs) conditioned medium (hMSCs-CM) can influence human embryonic stem cells (hESCs) chondrogenic differentiation. In this study, we hypothesized that conditioned medium (CM) from hESCs-derived MSCs in a sequential 3D-2D culture system could facilitate the induction of chondrogenesis in hESCs.

Materials and Methods: CM was collected from Yazd2 (hESCs; 46, XY) derived MSCs confluent cultures and stored at -20 °C. Yazd4 hESC line (46, XX) was induced for differentiation using EB formation as 3D culture into SD (spontaneously differentiation) and CM groups (differentiation using conditioned medium) for four days. Cell culture continued in a 2D (monolayer) culture system for both groups till day 14. Ultimately, chondrogenic differentiation was assessed by Alcian blue and masson′s trichrome staining at 4 and 14 days of differentiation, and quantitative real-time polymerase chain reaction (PCR) for *NANOG*, *MEOX1*, *SOX5*, *SOX6*, *SOX9*, *ACAN*, *COL2A1* and *RUNX2* genes for SD and CM groups on days 0 and 4.

Results: The gene expression profile for chondrogenic genes in the CM group was significantly more than the SD group ($p< 0.05$). Furthermore, chemical staining assessment illustrated a significant GAG and collagen II difference between the CM and SD groups at days 4 and 14 ($p< 0.05$).

Conclusions: Our findings would pave the way for creating an *in vitro* human chondrogenesis model for further studies in the developmental biology of articular cartilage tissue, which lend itself to cell-based therapy to cure joint diseases such as osteoarthritis.

*Corresponding Author: Stem Cell Biology Research Center, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. **Postal code:** 89168777391, **Tel:** +983538247085, **Email:** b.aflatoonian@ssu.ac.ir

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Introduction

Healing of damaged human joint cartilage does not occur spontaneously, leading to osteoarthritis [1]. Osteoarthritis is the most common joint disease characterized by the gradual degeneration of articular cartilage and affects millions worldwide annually. Different approaches were applied for osteoarthritis treatment. Due to the complications along with arthroplasty (joint surgery), cell-based therapies were considered, and stem cells were applied as an appropriate cell source for osteoarthritis cell therapy. Because of pluripotency, unlimited self-renewal, and human source, human embryonic stem cells (hESCs) can be used as a confident cell source for human articular cartilage regeneration [2, 3]. Initially, hESCs chondrogenic differentiation was induced using co-culture with chondrocytes in 2006 [4]. Chondrogenic differentiation of hESCs was confirmed by the expression of chondrogenic genes and markers and *in vitro* and *in vivo* (in severe combined immunodeficiency mice) tissue formation by differentiated cells.

Consequently, other studies verified the positive effect of the co-culture with chondrocytes on hESCs differentiation into chondrogenic progenitors [5-7]. On the other hand, most studies used defined culture conditions containing various growth factors as an effective method for chondrogenesis of hESCs [8-12]. It has been proven that conditioned medium (CM) obtained from chondrogenic cells provides an effective condition for introducing chondrogenic differentiation from hESCs [13]. Interestingly, CM from human mesenchymal stem cells (MSCs) showed a similar outcome on in vitro chondrogenesis using hESCs [14].

Furthermore, some studies highlighted the positive effect of sequential 2D-3D culture on the differentiation of hESCs toward chondrocytes [15]. This study investigated the effect of hESCs-derived MSCs conditioned medium and a sequential 3D-2D system on the induction of chondrogenesis in hESCs. Embryoid body formation was done from hESCs in SD (spontaneous differentiation) and CM groups (treatment with hESC-derived-MSCs conditioned medium), and then, the culture was followed by monolayer culture (2D culture) in both groups by day 14. Chondrogenic differentiation was evaluated by chemical staining and quantitative real-time polymerase chain reaction (PCR). This approach may be an effective way to enhance the differentiation of hESCs toward the chondrogenic lineage, which can be used to regenerate cartilage tissues in cell-based therapies.

Materials and Methods

Chemicals and medium

Dulbecco's Modified Eagle Medium / DMEM powder (Gibco, USA), Dulbecco's Modified Eagle Medium F12 (Gibco, USA), knock out-DMEM (Gibco, USA), fetal bovine serum (FBS) (Gibco, USA), Penicillin-Streptomycin (Gibco, USA), Gentamicin (Exir pharmaceutical company, Iran), knock out-serum

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replacement (Gibco, USA), Non-essential amino acid (100X) (Gibco, USA), basic fibroblast growth factor/ bFGF powder (Gibco, USA), L-Glutamine (Gibco, USA), β-mercaptoethanol (Sigma, USA), paraformaldehyde (Sigma, USA), glutaraldehyde (Sigma, USA), phosphate buffer saline/ PBS (Atocell, Austria), mitomycin C (Sigma, USA), alcian blue powder (8X) (Sigma, USA), hematoxylin (Merck, Germany), chromotrope 2R/ fast green (BioGnost, Croatia), acetic acid (Merck, Germany) DNAase treatment kit (Thermo scientific, USA), cDNA synthesis kit (Takara, Japan), real-time PCR kit (Takara, Japan), TRI reagent (Sigma, USA), SYBER Premix DimerEraser (Takara, Japan).

HESCs culture and maintenance

Yazd 4 hESCs line [2, 18] (46, XX, Stem cell biology research center, Yazd reproductive sciences institute, Shahid Sadoughi university of medical sciences, Yazd, Iran) were maintained in an undifferentiated state by culture on feeder layers of mitomycin-C-treated Yazd human foreskin fibroblasts #8 (YhFF #8, 46XY, Stem cell biology research center, Yazd reproductive sciences institute, Shahid Sadoughi university of medical sciences, Yazd, Iran) in knock out human embryonic stem cells (KOHES) medium at 37ºC and 5% CO2 in the air. The culture media consisted of Knock out DMEM supplemented with 20% (v/v) knock-out serum replacement, 1% L-Glutamin β-mercaptoethanol, 1% Non-essential amino acid (100X), and 0.2% bFGF. Also, the culture media used for the culture of YhFF#8 was made up of DMEM supplemented with 10% (v/v) FBS, 250 µl Penicillin-Streptomycin, and 500 µl Gentamicin. KOHES medium was changed every day. Every 5 days, undifferentiated hESCs colonies were cut into equal sizes through a mouth pipet and were passaged onto fresh YhFF#8 cells using one drop-one colony method [2].

HESCs-derived-MSCs conditioned medium preparation

Yazd2-derived-MSCs [16, 17] (46, XY, Stem cell biology research center, Yazd reproductive sciences institute, Shahid Sadoughi university of medical sciences, Yazd, Iran) were plated on a 250 mL flask (15×10^5 cells) in 12 mL DMEM supplemented with 10% (v/v) FBS, 250 µl Penicillin-Streptomycin, and 500 µl Gentamicin. After 4-5 days, when the cells were 80% confluent, the cells were split at a ratio of 1:2 on 250 mL flasks. The CM was collected, filtered by a 0.22 µm filter, and stored at -20°C for short-term storage for up to one week.

3D-culture Embryoid body (EB) formation

To induce differentiation, colonies were fragmented in big sizes using mouth pipet and cultured in EB medium comprised of Knock out DMEM supplemented with 20% (v/v) knock-out serum replacement, 1% L-Glutamin β-mercaptoethanol, and 1% non-essential amino acid (100X). EB formation was performed in two groups, including the SD group (spontaneously differentiation) and the CM group (differentiation via Yazd2-MSCs CM), for four days (Fig. 1). The media for the SD group contained 100% EB medium. The CM group was comprised of EB medium/ Yazd2-MSCs CM (1:1). The final volume of culture medium for both groups was 7ml, half

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of which was replaced with fresh medium every other day.

2D-culture

The culture was followed by 2D (monolayer) culture until day 14, and EBs from both groups were transferred to T25 flasks containing 3 ml DMEM F12 medium, including 3ml DMEM F12/ 10%FBS for the SD group, and DMEM F12/ 10%FBS+ Yazd2-MSCs CM (1:1) for CM group (Fig.1C and D). The medium was changed on alternative days. In order to prevent contact inhibition and cell contamination, cells in both groups were passaged at day eight at the ratio of 1:2 into T25 flasks containing the aforementioned media.

Quantitative real-time PCR

Quantitative real-time PCR was performed to assess the relative quantitative expression of *NANOG* as a pluripotency gene; *MEOX1* as a mesodermal gene; *SOX5*, *SOX6*, *SOX9*, *COL2A1*, *ACAN*, and *RUNX2* as chondrogenic genes and *GAPDH* as the housekeeping gene at days 0 and 4. At first, primers were sequenced, and the appropriate annealing temperature was determined by gradient PCR. After cell harvesting at day four from both groups, RNA extraction was done by TRI reagent standard protocol supplied by the manufacturer. To remove genomic DNA contamination from samples, 1µg of extracted RNA was treated with DNase I, and 500 ng of treated RNA was used for cDNA synthesis through Prime Script RT Reagent kit. Finally, quantitative real-time PCR was done using the RotorGeneQ system (Qiagen) with SYBER Premix DimerEraser for 40 cycles. Each cycle consisted of these times and temperatures: 95 °C (5 sec), annealing temperature (30 sec), 72 °C (30 sec). The primers are mentioned in Table 1.

Table 1. The list of the primers used for Q-PCR

Gene name	Forward Primer (3'-5')	Reverse Primer (5'-3')	Annealing Temperature	Product Size (bp)	Accession Number
GAPDH	CAAGAGCACAAGAGGAAGAGAGAG	TCTACATGGCAACTGTGAGGAG	64°	103	NM_002046.7
NANOG	CCCCAGCCTTTACTCTTCCTA	CCAGGTTGAATTGTTCCAGGTC	60 °	97	NM_024865.4
MEOX1	ACTCGGCTCCGCAGATATGA	CCAGGTTGAATTGTTCCAGGTC	60 °	103	NM_001040002.2
SOX5	GTAGTGACCCTTACCCTGTTCAG	TGCAGTTGGAGGTGGCCTA	64°	88	NM_006949.6
SOX6	GCAGTGATCAACATGTGGCCT	CGCTGTCCCAGTCAGCATCT	64°	120	NM_001145811.2
SOX9	AGACAGCCCCCTATCGACTT	CGGCAGGTACTGGTCAAACT	64°	108	NM_000346.4
COL2A1	TAAGGACGTGTGGAAGCCGGA	GGCTGAGGCAGTCTTTCACG	64°	104	NM_001844.5
ACAN	ACAAGGTCTCACTGCCCAACT	GCCTTTCACCACGACTTCCAG	64°	150	NM_001135.3
RUNX2	GTCCCCGTCCATCCACTCTA	CAGAGGTGGCAGTGTCATCAT	64°	110	NM_001369405.1

Chemical staining

Alcian blue was carried out to detect glycosaminoglycan/ GAG secreted by chondroprogenitors. 4-day EBs and the differentiated cells at day 14 in SD and CM groups were harvested and fixed with 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes at room temperature. Afterward, fixed cells were stained with alcian blue (1% w/v, pH 2.5) for 90 minutes. Masson's trichrome staining was used to monitor the presence of collagen in EBs in different groups.

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EBs were fixed in 4% (v/v) glutaraldehyde and stained by hematoxylin solution for 7 minutes. After removing hematoxylin from the cells by PBS, samples were stained by chromotrope 2R/ fast green and washed with 1% (v/v) acetic acid for 10 seconds [18]. YhFF#8 cells were used as the negative control group in both procedures. After cell washing with PBS, cells were examined by an inverted microscope, and imaging was performed. Finally, the number of stained cells was calculated by ImageJ software (1.52v).

Statistical analysis

The statistical analysis for real quantitative time-PCR and chemical staining data was performed by student′s t-test (IBM SPSS Statistics 20 software and GraphPad Prism 9.3.1). A $p < 0.05$ was considered statistically significant.

Results

HESCs maintenance and 3D-2D culture system

The hESCs were expanded using the one drop-one colony method on inactivated YhFF#8 feeder layers (Fig. 1). EB formation was done in the SD and CM groups for four days to induce chondrogenic commitment in hESCs. However, EBs in the CM group appeared to be similar to the SD group in shape and size, and no significant difference was observed between both groups (Fig.2). The culture had been followed through a 2D culture system in both groups by day 14. As Fig. 3 illustrates, at the end of the culture period (day 14), cells in both groups exhibited a flat, small, spherical shape, which is the typical morphology of chondrocytes. Meanwhile, some cells in the SD group demonstrated a fibroblast-like morphology.

Quantitative real-time PCR

Comparing gene expression profiles, significant differences were observed in the expression of mesodermal and chondrogenic genes between the SD and CM groups, as it is shown in Fig. 4. In contrast, in the CM group, the expression of *MEOX1*, *SOX5*, *SOX6*, *SOX9*, *ACAN*, and *COL2A1* genes were significantly higher than the SD group (Fig. 4B, C, D, E, F, $P<0.001$, and Fig. 4G, $p<0.01$), the expression of *RUNX2* did not show any significant difference between the SD and CM groups ($p>0.05$, Fig. 4H). However, these genes almost showed no expression in undifferentiated hESCs. On the other hand, *NANOG* mRNA was highly expressed in undifferentiated hESCs and, after four days, saw a significant decrease (Fig. 4A).

Chemical staining

Chemical staining was applied for semi-quantitative assessment of hESCs chondrogenic differentiation at days 4 and 14. According to fig.5, the results of alcian blue staining showed that the presence of GAG in the CM-EBs was significantly higher than in the SD-EBs (Fig. 5A, B, $p>0.05$). Similarly, Masson's trichrome staining data proved the higher presence of collagen II secreted by the EBs in the CM group (Fig. 5D, E, $p>0.01$). Turning to the 2D cultured cells, what stands out from fig. 6 is that the presence of mentioned markers in the CM group was considerably higher than in the SD group ($p>0.05$). These markers were not observed in YhFF#8 as the negative control group (Fig. 4C, F, and Fig. 5C, F).

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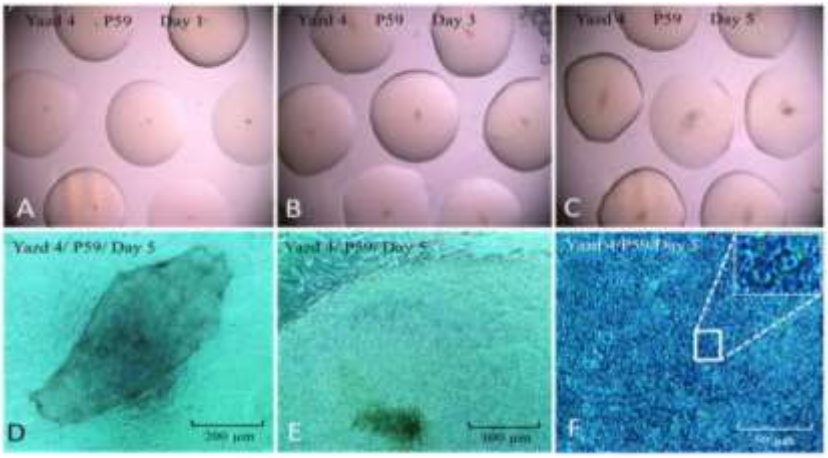

Fig. 1. **Undifferentiated Yazd4 hESC colonies (passage number 59).** A: One day after passage; B: Three days after passage; C: Five days after passage. A Yazd4 hESCs colony in a drop with 4x (D) 10x (F) 20x (E; the high nucleocytoplasmic ratio and several nucleoli (black spots) have been shown in the square.)

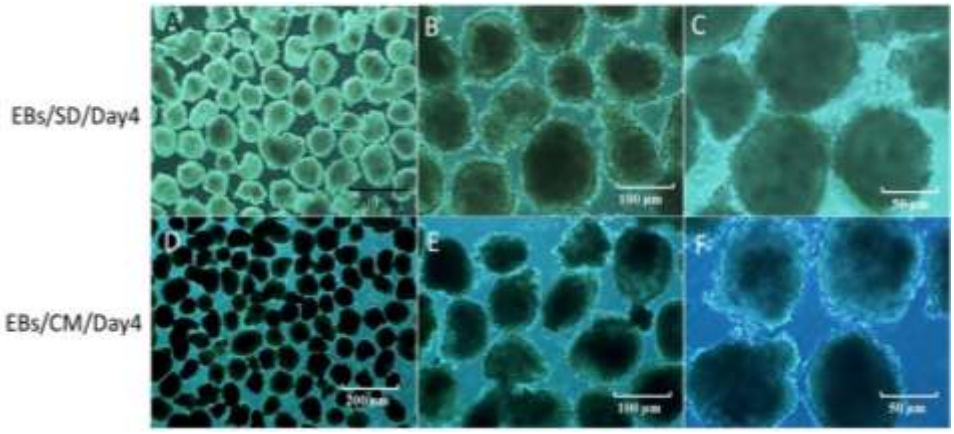

Fig. 2. **Shape of EBs in both groups was similar.** A-C: EB formation in SD group, scale bar: 200 µm (A), 100 µm (B), 50 µm (C). D-F: EBs in CM group, scale bar: 200 µm (D), 100 µm (E), 50 µm (F)

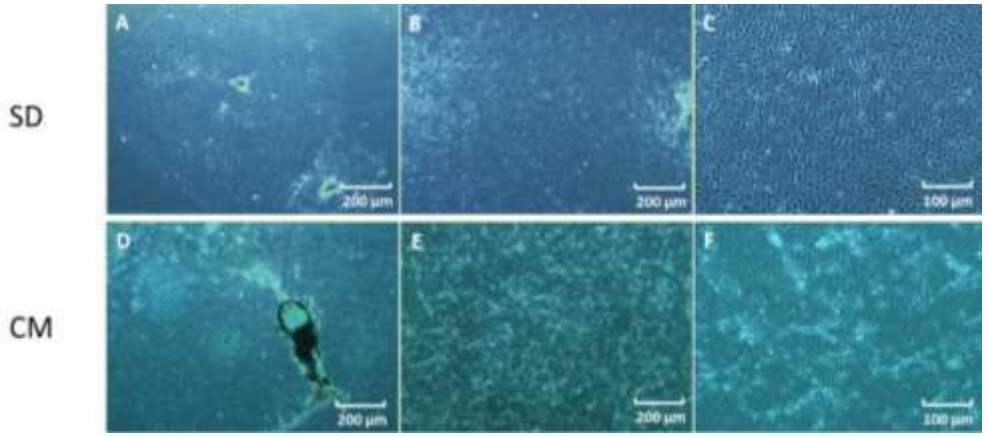

Fig. 3. 2D culture. A-C: monolayer culture in the SD group. A: Cells before passage at day 8 (, 200 µm), B, C: cells after passage at day 14 (B: 200 µm, C: 100 µm), D-F: monolayer culture in the CM group. D: Cells before passage at day 8 (, 200 µm), E, F: cells after passage at day 14 (B: 200 µm, C: 100 µm).

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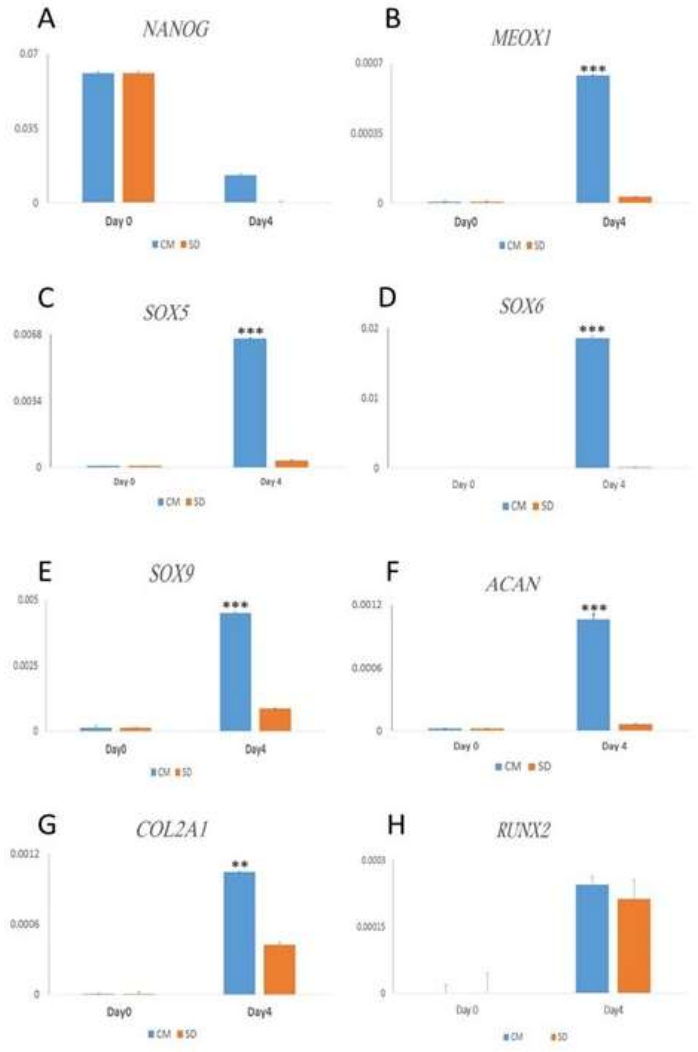

Fig. 4. Gene expression profile at days 0 and 4. The gene expression of *NANOG* (A), *MEOX1* (B), *SOX5* (C), *SOX6* (D), *SOX9* (E), *ACAN* (F), *COL2A1* (G), and *RUNX2* (D) was compared between undifferentiated hESCs and EBs (on day 4), in both CM and SD group (*p< 0.05, **p< 0.01, ***p< 0.001).

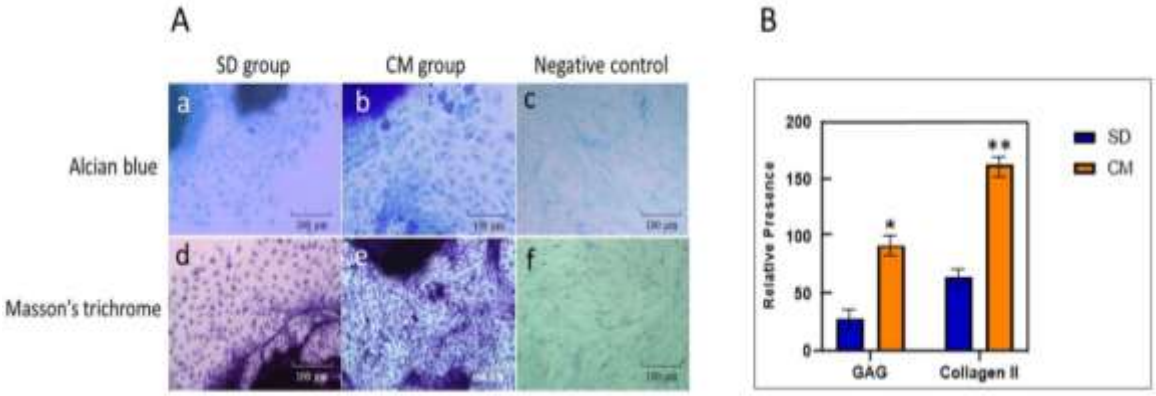

Fig. 5. A. Alcian blue and Masson′s trichrome staining for 3D culture. Alcian blue staining for SD group (a), for CM group (b), and YhFF#8 cells as negative control (c). Masson′s trichrome staining for SD group (d), for CM group (e), and YhFF#8 cells as negative control (f). Scale bars: 100 µm. B. Enhanced presence of the chondrogenic markers in the CM group at day 4 in compared with untreated EBs in the SD group (*p< 0.05, **p< 0.01).

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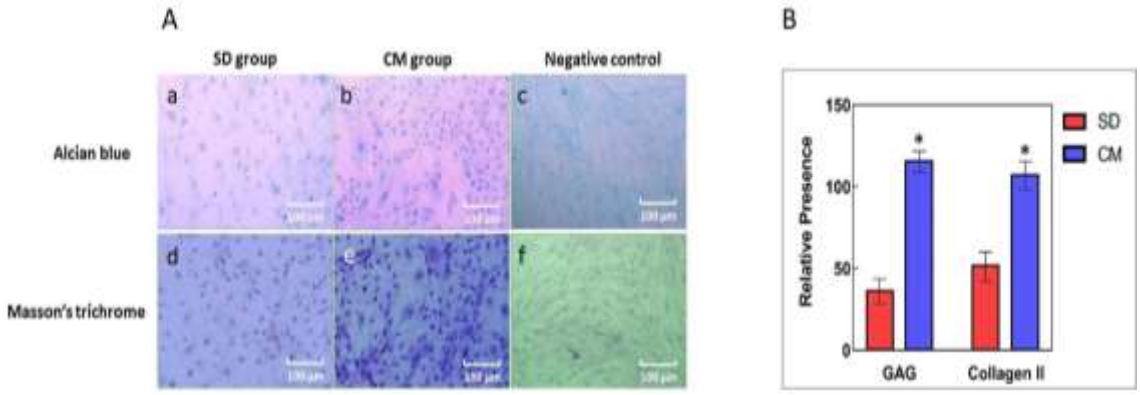

Fig. 6. A. Alcian blue and Masson′s trichrome staining for 2D culture. Alcian blue staining for SD group (a), for CM group (b), and YhFF#8 cells as negative control (c). Masson′s trichrome staining for SD group (d), for CM group (e), and YhFF#8 cells as negative control (f). Scale bars: 100 µm. B. Enhanced presence of the chondrogenic markers in the CM group at day 4 in compared with untreated EBs in the SD group (*P< 0.05).

Discussion

Preceding research reported that the conditioned medium from human MSCs can support chondrogenesis from hESCs [14]. Furthermore, some studies claimed that EB formation followed by monolayer culture could be an effective culture method for chondrogenesis induction [15]. In this report, we investigated the effect of hESCs-derived-MSCs CM and sequential 3D-2D culture on hESCs chondrogenic differentiation. Since the Yazd4 hESCs cell line was initially derived in a xeno-free culture system (on human foreskin fibroblast feeder layers), using this cell line could lay the ground for the clinical use of Yazd4-derived chondrocytes in the future. EBs generated from Yazd4 hESCs were similar in both SD and CM groups. However, in contrast to the SD group exhibiting a heterogeneous cell population in the 2D culture, the majority of cells in the CM group had a chondrocyte-liked shape, which morphologically resembles the monolayer cultured cells in the Bay et al. study [15]. Yodmuang et al. [13] showed that the *NANOG* pluripotency gene was highly expressed at day 0 in undifferentiated hESCs. However, the expression of this gene plummeted at day 4 in both groups.

Interestingly, EBs in the CM group expressed *NANOG* at a low level, whereas this gene was not expressed in the SD group. The most probable explanation for this phenomenon is that hESCs-derived-MSCs CM causes *NANOG* overexpression in differentiated cells, which could improve chondrogenic commitment [20]. The gene expression profile of the CM-treated EBs revealed a significant increase in the expression of mesodermal and chondrogenic genes compared with the SD group, which confirms the results of the Lee et al. study [14]. By contrast, the expression of *RUNX2* was not as high as other genes and did not show a significant difference between the mentioned groups. The reason behind this could be that since this gene is primarily expressed in mature chondrocytes *in vivo* [1, 19], it is presumed that using hESCs-derived-MSCs CM can only

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induce the early stages of chondrogenesis. Turning to chemical staining data, Liu et al. showed that the presence of GAG and collagen II in the CM-treated EBs was significantly higher than in the EBs differentiated spontaneously [21]. Likewise, the higher presence of the aforementioned markers in the CM group on day 14 confirms the positive impact of hESCs-MSCs CM on the chondrogenic commitment of hESCs.

Conclusion

In summary, our data show that hESCs-derived-MSCs CM has the potential to induce chondrogenesis in hESCs and can be an efficient and cost-effective method for producing chondroprogenitors, which are considered valuable sources for cell therapy of damaged articular cartilage. These results could lay the ground for a better understanding of various aspects of chondrogenesis, further studies in developmental biology, and clinical research in respect to finding possible osteoarthritis therapies. Undoubtedly, further studies are required to prove the definitive effect of this method. It is recommended to use defined chondrogenic medium culture as the positive control. Also, it is suggested to purify chondroprogenitors from the CM group, culture them in a defined chondrogenic medium, and investigate the tissue formation potential of mature chondrocytes *in vivo* and *in vitro*.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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