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Mixed enzymatic-explant protocol for isolation of mesenchymal stem cells from Wharton's jelly and encapsulation in 3D culture system

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ABSTRACT

We report combination of explants and enzymatic protocol as mixed enzymatic-explant procedure to faster extraction of MSCs from WJ. Umbilical cords (UC) were collected from Imam Khomini Hospital. For explant outgrowth, 6 - 9 pieces of WJ were transferred onto tissue culture flask and waited for attachment. For mixed enzymatic-explant, 1 cm^3 pieces WJ were placed in enzymatic cocktail comprising 4 mg/ml Collagenase Type I and 1 mg/ml Hyaluronidase and 0.1% trypsin-EDTA. Then isolated cells were analyzed for surface cell markers such as CD73, CD31. Isolated 1.0 × 10^6 MSCs/ml were encapsulated in alginate hydrogel. Cells with MSCs phenotype were isolated from mixed enzymatic-explant and explant procedures within 24 - 48 hrs and 7 - 10 days, respectively. Both of procedures were shown to form clumps and colonies with dense centers. Phenotypic changes gradually appeared as round cell in UC pieces into homogeneous spindle-shaped and typical fibroblast-like shape cells. By using flow cytometery MSCs showed positive for CD73, and negative for CD31. the morphology of viable MSCs in the beads did not significantly show a different morphology pattern before and after the bead formation process. These findings are indicated that when mixed enzymatic-explant procedure is performed MSCs can be isolated faster and much higher from WJ. These finding is important in comparing with time consuming explants culture for isolation of MSCs.

Keywords: Mesenchymal Stem Cells; Isolation; Flow Cytometery; Alginate

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1. INTRODUCTION

Stem cells are precursor cells which have two distinct characteristics: self-renewal and differentiation these unique cells and can be classified according to developmental potential (pluripotential, multipotential, and unipotential cells) and origin (embryonic stem (ES) cells, embryonic germ (EG) cells, embryonal carcinoma (EC) cells, trophoblast stem (TS) cells, and foetal stem cells). They have the potential to develop into many different cell types [1].

Mesenchymal stem cells (MSCs) are adult stem cells [2] and it is well accepted that umbilical cord a source for mesenchymal cells. Therefore, many studies investigated on isolation and expansion of MSCs from human UC tissue, namely the WJ [3].

Because UC is a rich source of MSCs, many groups reported isolation of MSCs from different parts of WJ matrix with various derivation protocols. Based on these reports MSCs isolated as a perivascular stem cell from the perivascular regions around the blood vessels [4], as a sub-amnion stem cell from the inner amnion membrane [5], as a human amnion membrane stem cell from the outer amnion membrane [6], as a human umbilical cord matrix stem cell from the intervascular compartment [7] and as a human Wharton's jelly stem cell; WJSC from the WJ [8].

Several studies reported 3D culture of MSCs in various scaffold. One of the most frequent biomaterial to fabricate hydrogel scaffold and encapsulation is alginate, which extracted from seaweeds. During cell encapsulation living cells entrapped within hydrogel to provide

three dimensional environments [9]. In recent year, studies reported encapsulation of MSCs in scaffolds such as alginate [10,11]. Cell encapsulation have several advantages such as protection cells/tissue fragments from immunological reaction, maintain good viability, the immobilization of cells within microbeads, allows high-density cell culture and free exchange of nutrients, oxygen, and bioactive products, increase the function of mature cells, but one of major disadvantages of alginate is poor mechanical property.

Various protocol of MSCs population extraction have been reported from WJ such as explants and enzymatic procedures and results were controversial [12-15]. Therefore, the objective of this study is to introduce isolation of MSCs from WJ suggested new method. We report combination of explants and enzymatic protocol as mixed enzymatic-explant to faster extraction MSCs from Wharton's jelly. We compared the morphological changes of isolated MSCs in two different 2D and 3D culture in alginate scaffold.

2. MATERIAL AND METHODS

2.1. Materials

Phosphate-buffered saline (PBS), fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), antibiotic (penicillin–streptomycin), alginate and trypsin, HEPES, L-glutamine, Collagenase Type I, Hyaluronidase, trypsin-EDTA, NaCl and $CaCl_2$ were purchased from Sigma-Aldrich (MO, USA). All chemicals were used directly without further purification. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

2.2. Ethic Approval

The study protocol was approved by the Ethics Committee of Ahvaz Jundishapour University of Medical Sciences (AJUMS) and all procedures were performed according to ethical committee approval. UC cells were harvested from the umbilical cords of healthy full-term infants born by cesarean section (C/S). Before collection of UC informed consent received from the mothers.

2.3. Collection and Transport of UCs from Hospital to Laboratory

Protocol design for this study is presented in **Figure 1**. According to this protocol, the work was divided into five steps; isolation phases (by explant or mixed enzymatic-explant procedures), proliferation of MSCs, flow-cytometry with negative and positive surface markers, two dimensional and three dimensional cultures and evaluation of MSCs (**Figure 1**).

All of UC were collected from Obstetrics Department of Ahvaz Jundishapour University of Medical Sciences (AJUMS) and approximately, 20 cm length pieces cut from each UC. Immediately after C/S, the cords were transferred to sterile containers containing sterile transport medium (DMEM (low glucose) containing 100 U/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B. and stored at 4˚C for 2 - 6 hrs before tissue processing. Collected UC were transported to laboratory of Cellular and Molecular Research Center (CMRC), Department of Anatomical Sciences, Faculty of Medicine, Ahvaz Jundishapour University of Medical Sciences (AJUMS).All the procedure of collection and transport of C/S were performed under sterile conditions.

2.4. Extraction of WJ

Isolation and propagation of MSCs from above small pieces from WJ were carried out according to the protocol is found elsewhere. At least two investigators cooperated in tissue processing and Wharton's jelly dissection. MSCs isolation were performed in cold, sterile dissection solution consisting of 1% antibiotic solution in PBS to give 100 U/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B. Briefly, in laminar hood and under sterile conditions, each piece of umbilical cord was cut into smaller 2 - 4-cm length pieces. The cord was washed gently with carrying solution three times and squeezed with curved forceps to remove trapped blood within the umbilical blood vessels. The washing was repeated until remove any blood or blood clots. At the external surface of each piece, horizontal sections are engraved with a sterile scalpel then cut open lengthwise

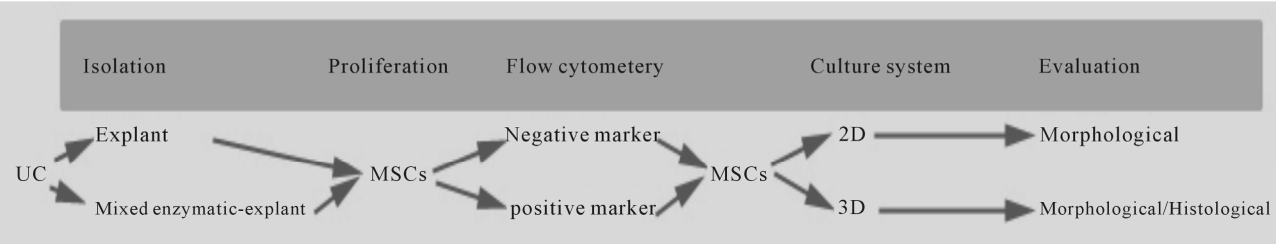

Figure 1. Design study. Steps of the work divided into several phases such as isolation (by explant or mixed enzymatic-explant procedures), proliferation of MSCs, flow cytometry with negative and positive surface markers, two dimensional and three dimensional cultures and evaluation of MSCs.

with sterile scissors and forceps and cut the outer sheath of UC sections to extract its inner surface containing the WJ. Then, the exposed vein and arteries were removed by pulling with forceps and peeled away from the WJ. Extracted WJ was cut into 3 - 5-mm pieces with a scalpel and washed again with sterile dissection solution [12-15] (**Figure 2**).

2.4.1. Isolation of MSCs with Explants Procedure

For explant outgrowth, 6 - 9 pieces were transferred onto tissue culture 25-cm^2 T-flasks and were left undisturbed for 3 - 5 minutes until attachment onto flask. Then, complete culture medium (CCM) composed of DMEM (low glucose) with 2 mM L-glutamine, supplemented with 20% fetal bovine serum, 100 U penicillin/streptomycin was added. The culture flask was left undisturbed for 3 - 4 days and maintained at 37˚C in a humidified atmosphere containing 5% CO_2. The medium changed every 2 - 3 days thereafter [12-15] (**Figures 2** and **3**).

2.4.2. Isolation of MSCs with Mixed Enzymatic-Explant Digestion

After removing blood vessels and outer sheath of UC to allow only the WJ to come into direct contact with the enzymatic solution, extracted WJ cut into approximately 1 cm^3 pieces and washed with PBS. WJ, were then placed in 50 ml falcon tube containing LG-DMEM medium with enzymatic cocktail comprising 4 mg/ml Collagenase Type I and 1 mg/ml Hyaluronidase and incubated for 1hr. followed by 0.1% trypsin-EDTA for 30 minutes at 37˚C in a humidified atmosphere containing 5% CO_2 to loosening up of the WJ without complete digestion (**Figure 4**). After the incubation period, the WJ pieces were washed with PBS solution and transferred to a new 50 ml falcon tube and procedure of explant outgrowth was repeated (**Figure 4**). The digested suspendsion was diluted 1:2 with PBS solution and passed through a 70 μm nylon filter (Falcon, BD, Oxford, UK), diluted with LG-DMEM, and centrifuged at 300 g for 10 min. Supernatant discarded and cell pellets resuspended in culture medium (CCM) composed of DMEM (low glucose) with 2 mM L-glutamine, supplemented with 20% fetal bovine serum, 100 U penicillin/streptomycin.

The entire tissue flask containing the WJ pieces in either above mentioned procedure was left undisturbed at 37˚C in a humidified atmosphere containing 5% CO_2 until migration of MSCs from WJ [12-15].

Figure 2. Isolation of MSCs by explant procedure. (A) Transport of UC from hospital in cold sterile environment; (B) Transfer of UC into petri dish under laminar hood; (C)-(E) Dissection and separation of outer sheath and blood vessels from WJ. Arrow indicates matrix of WJ, outer sheath and blood vessels; (F) Isolated WJ pieces.

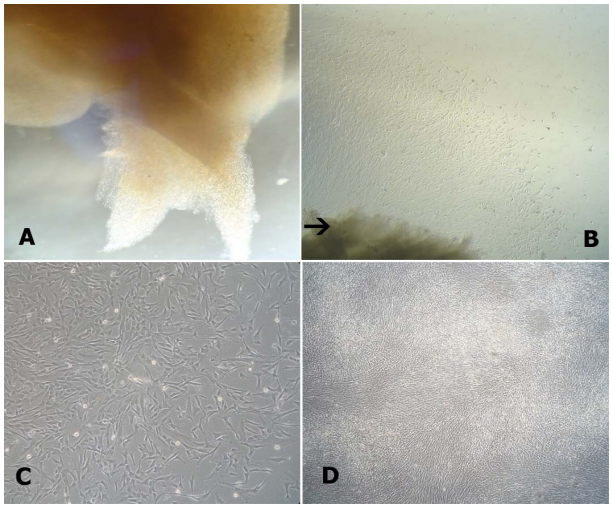

Figure 3. Explant culture of WJ. (A) A piece of WJ; (B) Migration of MSCs from WJ (arrow); C) Culture of fibroblast like MSCs; D) Confluency of culture MSCs in 2D.

2.5. Cell Expansion

Cells were allowed to adhere to culture flask and non-adherent cells were removed by changing the medium. When the adherent cells reached confluence, covering 70% - 80% of culture flask, the cells were washed once with PBS and trypsinized with 0.25% trypsin-EDTA. After detachment of the adherent cells, suspension centrifuged at 200 - 300 g for 10 min, supernatant discarded, cell pellets washed in medium. Then were resuspended

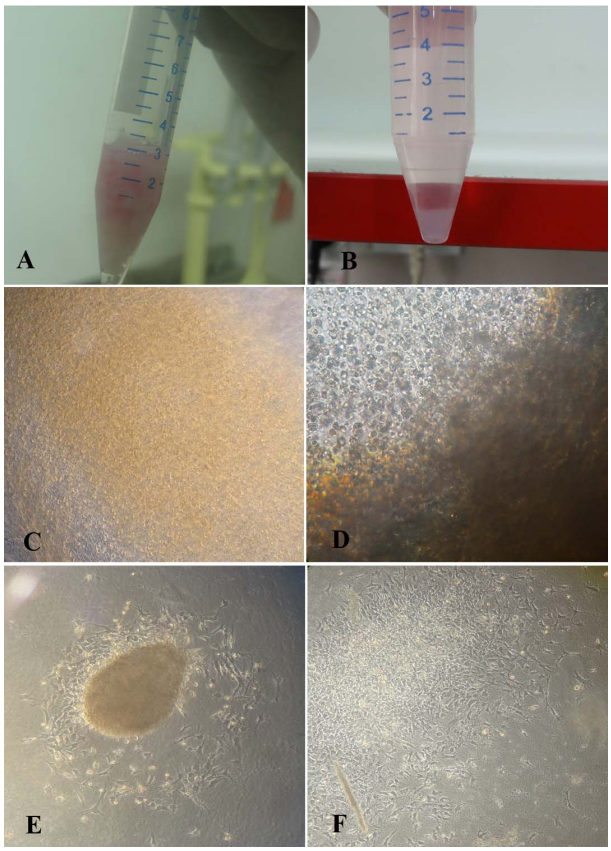

Figure 4. Mixed enzymatic-explant isolation of MSCs from WJ. (A) Pieces of WJ digested with enzyme; (B) Pellet of MSCs; (C), (E) Pieces of WJ after enzyme digestion; (D), (F) Migration of round MSCs from WJ and change morphologically into fibroblast like cells.

in medium and transferred to new culture flask at a density 250 × 10^3 cells/cm^2. During subculture, they were passaged every 5 days; medium was replaced every 3 days. Expansion medium consisted of DMEM (low glucose) with 2 mM L-glutamine, supplemented with 20% fetal bovine serum, 100 U penicillin/streptomycin. Adherent cells were cultured until they reached 80% - 90% confluence [12-15]. For further passaging above procedures were conducted again.

2.6. MSCs Morphology Analysis

The MSCs cultures were routinely visualized using an inverted research microscope IX50 (Olympus, Japan) and the phenotype and growth of primary and passaged cells were monitored Photomicrographs were taken with a digital camera model of Cyber-shot DSC-HX9V (Sony, Japan) under inverted phase-contrast optics.

2.7. Biomarkers Detection of MSCs by Using Flow Cytometry

MSCs were analyzed for surface cell markers by Flow cytometry as described in the Supplemental Data. Cells were trypsinized. And total of 5 × 10^5 cells were resuspended in PBS and 5% FBS incubated with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies against CD73, CD31 and IgG (ebioscince, USA) for 30 min at dark room at 4˚C. Excess antibody was removed by washing and detection of PE and FITC labeling was accomplished on a Dako Glaxy flocytometer. Mouse isotype-matched Antibody (Ab) were used as controls (ebioscince, USA) [12-15].

2.8. Cell Encapsulation of MSCs in Hydrogel Scaffold

For alginate hydrogel scaffolding, alginate of 2.0% w/v were prepared as previously described by adding alginic acid sodium salt from brown algae, into 0.15 M NaCl, and 0.025 M HEPES in deionized water by stirring. The solution was then sterilized via 0.45-nm filter [14].

Isolated MSCs (1× 10^6 cells/ml) were resuspended in 2 - 5 ml prepared hydrogel and then slowly expressed through a 22 gauge needle into a 102 mM $CaCl_2$ solution. The hydrogels containing encapsulated hMSC were allowed to polymerize for a period of 10 minutes in the $CaCl_2$ solution. Next, microcapsules were washed 2 - 3 times 10 volumes of 0.15 M NaCl. The alginate/MSCs constructs were finally placed in culture flask with complete culture medium composed of DMEM (low glucose) with 2 mM L-glutamine, supplemented with 20% fetal bovine serum, 100 U penicillin/streptomycin and with controlled environmental conditions (37˚C, 5% CO_2, 95% O_2 and 95% humidity) [10,11].

2.9. Histological Evaluation

In the end of MSCs 3D culture in hydrogel scaffold, the constructs were fixed in Bouin's fixative solution, dehydrated through a series of graded alcohols, cleared with xylene, and embedded in paraffin wax. Five to seven-micrometer sections were cut, and then stained with hematoxylin & eosin.

2.10. Statistical Analysis

Data were given as mean ± standard deviation and were statistically analyzed. The difference between the mean was considered significant when $p < 0.05$.

3. RESULTS

3.1. Isolation of MSCs with Explant and Mixed Enzymatic-Explant

Cells with a MSCs phenotype were isolated from mixed enzymatic-explant within 24 - 48 h of collection, while in explant procedure through 7 - 10 days after collection,

MSCs phenotype cells were isolated. Therefore, when compared isolation of MSC from explant culture was much slower to the enzymatic-explant cultures (2 ± 8.7 days compared to 15 ± 9.9 days, respectively) which led to confluence was reached much slower compared to the same enzymatic cultures. Although cell growth increased with time in enzymatic-explant culture, the increases were not as high as for with explants culture. Also enzymatic cultures produced MSCs at high efficiency (**Figures 3** and **4**).

3.2. Cell Expansion

There was no difference between explant and a mixed enzymatic-explant culture in proliferation after initial cell isolation either 24 - 48 hrs. In enzymatic-explant culture or 7 - 10 days in explant culture. Both of procedures were shown to form clumps and colonies with dense centers. These colonized populations were observed at the early stages of culture. Cells were cultured and maintained until passage 7 - 8 (**Figures 3** and **4**).

3.3. MSCs Morphology Analysis

Phenotypic changes gradually appeared as round cell in UC pieces into homogeneous spindle-shape and typical fibroblast-like shape cells which continued to proliferate. When these spindle-shaped cells appeared in culture, colonies formed thereafter. Although hMSC maintained their characteristics such as the fibroblastic morphology over the passages, primary and first-passage cultures displayed an epithelioid or short fibroblastic-like phenoltype but in further passages, cell transformed into long spindle-shaped fibroblast-like cells (**Figure 5**).

3.4. Immunophenotypic Characterization of MSCs

The immunophenotype of the WJSC was similar in both enzymztic and explant and cultured stem cells were positive for general mesenchymal markers such, CD73, but negative against hematopoietic markers, CD31 (**Figure 1**) although there were differences in growth rates. Therefore these data suggested that the UC derived cell is one of the MSCs populations similar to those from bone marrow or adipose tissue (**Figure 6**).

3.5. 3D Culture

Homogenous alginate beads successfully produced. Morphology of viable MSCs in the beads did not significantly revealed a different morphology pattern before and after the bead formation process. They had round shaped similar to round shape of stem cells in UC.

After 7 - 10 days of culture, the microscopic pictures suggested formation of three-dimensional cell aggregates

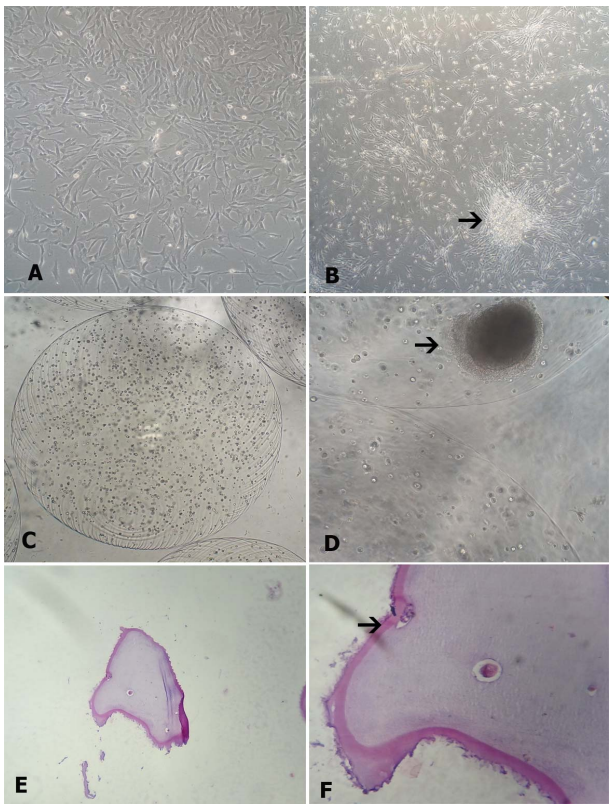

Figure 5. Comparison of cell morphology in 2D and 3D culture system. (A) Adherent fibroblast like cell in 2D culture; (B) Clump formation of MSCs in 2D culture (arrow); (C) Round MSCs within of a alginate bead; (D) Clump formation of MSCs in 3D culture (arrow); (E) Histological staining of an alginate bead; (F) MSCs in alginate hydrogel (arrow).

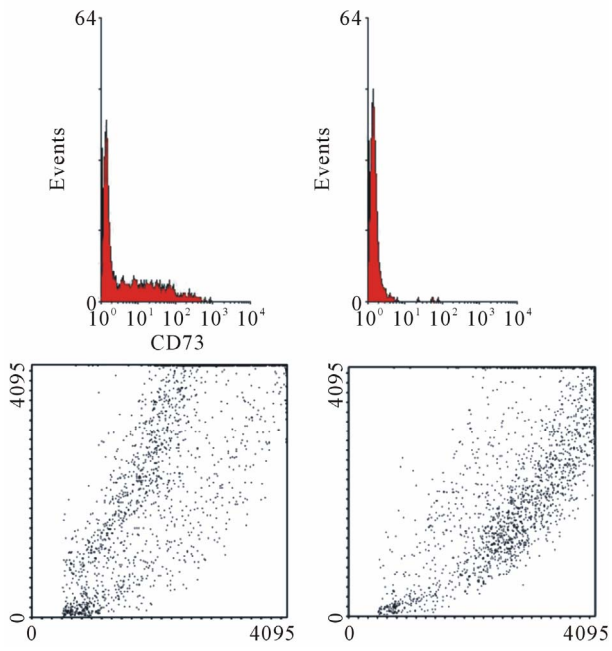

Figure 6. Flow cytometry for MSC surface markers. CD73 is shown in left graphs and CD31 is shown in right graphs.

as mounds, and the mounds in 3D were larger in size and greater in numbers compared with 2D (**Figures 5** and **7**).

3.6. Histological Evaluation

Typical histological appearance of sections revealed evidence of round cells with clear cytoplasm and nucleus which aggregated in some part of scaffold.

4. DISCUSSION

In present study, we examined isolation of MSCs from umbilical cord with different protocols: explants and mixed enzymatic-explant procedure. Results indicated that numbers of adherent MSCs in mixed explants-enzymatic protocol were much higher and faster that explants procedure.

In present study we used 4 mg/ml Collagenase Type I and 1 mg/ml Hyaluronidase and incubated for 1 hr. followed by 0.1% trypsin-EDTA for 30 minutes.

In literature different protocols have been reported to isolation of MSCs from UC with different findings. Investigators examined different dose and time: 2.7 mg/mL collagenase type I, 0.7 mg/mL hyaluronidase 2.5% trypsin, 3 h in PBS [15], 0.075% collagenase type II for 30 min, 0.125% trypsin for 30 min [16], collagenase type B (1 μg/mL) in DMEM-Ham's F12 (1:1), 10% FBS for 4 h [17], collagenase, hyaluronidase, trypsin, 45 - 60 min [7], collagenase 1 mg/mL (PBS) for 18 - 24 h [4], enzyme cocktail: 4 mg/mL BSA, 4 mg/mL collagenase, 1 mg/mL hyaluronidase, 0.1 mg/mL trypsin inhibitor and 200 mg/mL DNAse II [18], collagenase for 18 h, washed, 2.5% trypsin for 30 min with agitation.

In our study, isolated MSCs expressed strength mesenchymal markers such as CD73, while there negative against hematopoietic markers, CD31. Therefore these data suggested that the UC derived cell is one of the MSCs populations similar to those from bone marrow or adipose tissue. Our finding is consistent with other studies [19-21].

When MSCs migrated from WJ into culture flask, they have round shape cell morphology and changed morphological into spindle-shaped and fibroblast like cells. These features were observed by several studies, and they found morphology of MSCs similar to our findings [15,16,22].

In present study, encapsulated MSCs in alginate hydrogel had round shaped similar to round shape of stem cells in UC. Our finding is consistent study of Penolazzi *et al.* 2010 that "The microencapsulation procedure did not alter the morphology and viability of the encapsulated MSCs" [10].

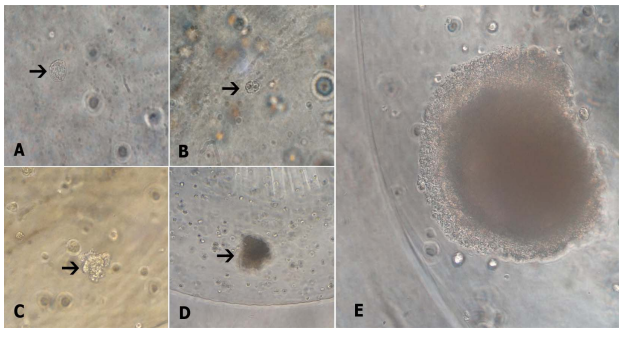

Figure 7. Clump formation of MSCs in 3D culture. (A) Round MSCs within alginate hydrogel (arrow); (B) mitosis of MSCs; (C)-(D) cluster formation MSCs as spheroid body.

5. CONCLUSION

These finding indicated that when mixed enzymatic-explant procedure is performed MSCs can be isolated faster and much higher from Wharton's jelly. These findings are important in comparing with time and consuming explants culture which for isolation of MSCs.

6. ACKNOLEDGEMENTS

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ABBREVIATIONS

Mesenchymal stem cells (MSCs), WJ (Wharton's jelly), Umbilical cords (UC), embryonic stem (ES) cells, embryonic germ (EG) cells, embryonal carcinoma (EC) cells, trophoblast stem (TS), Wharton's jelly stem cell (WJSC), Phosphate-buffered saline (PBS), fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), ethylenediamine tetraacetic acid (EDTA), cesarean section (C/S), complete culture medium (CCM), phycoerythrin (PE), fluorescein isothiocyanate-conjugated (FITC) antibodies.

