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## Viability and Morphology of Human Dental Pulp Stem Cells in The Presence of Citrus Pectin

Research Article

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### Abstract

Pectin is a galacturonic acid rich heteropolysaccharide which regulates plant cell metabolism. Plenty of fresh fruits and fruit pomaces from fruit juice production can be used as a raw material in commercial pectin production. Pectin occupies a large global market size especially in food industry and the utilization of waste materials for obtaining pectin molecules as a high value-added product makes it very favorite industrial material. Besides food industry, pectin is gaining attention in tissue engineering and drug development studies. In this study, the effects of citrus pectin on viability and morphology of human dental pulp stem cell (hDPSC) were investigated. The cells were cultured in the presence of pectin in culture medium (0.43, 0.85 and 1.7 mg/mL) for eight days. Resazurin application and MTT assay were applied on day one and eight for cellular viability. Cellular morphology was investigated by invert phase contrast microscope, live/dead cell staining and F-actin/nucleus immunofluorescence staining. MTT analysis results indicated that the viability of hDPSCs decreased significantly due to dissolved pectin in culture medium at applied concentrations. There was no significant morphological difference in the cells under invert phase contrast microscope and no significant staining difference in live/dead cell staining images. On the other hand, F-actin/nucleus staining showed that there were some condensed and crescent cell nuclei in the pectin applied groups when compared to the control groups which may be related to apoptosis. In conclusion, the viability of hDPSCs decreased and crescent cell nuclei formation was observed due to the presence of citrus pectin in the cell culture medium.

**Keywords:** Pectin, Human Dental Pulp, Mesenchymal Stem Cell

### 1. INTRODUCTION

Pectin is a complex plant cell wall polysaccharide which is responsible for cell growth, differentiation and defense mechanism. Pectins, as a biomacromolecule, mainly consist of galacturonic acid and rhamnogalacturonan I and substituted by rhamnogalacturonan II (RG-II), and xylogalacturonan (XGA) [1, 2]. They are divided in two groups according to the degree of esterification. High methoxy (HM) pectins

have esterification degrees above 50% and low methoxy (LM) pectins have esterification degrees below 50% [3-5].

Pectin is a widely used stabilizer and gelling agent in food industry and has expanding market share in cosmetics and pharmaceuticals [6]. It is obtained from various sources as citrus peel (85%), apple, sour cherry pomace and black cherry tomato [7-11]. The main pectin extraction method is based on acid hydrolysis in hot water [1]. There are some examples for different types of pectin extractions: pectin extraction from grapefruit (*Citrus paradisi* Macf.) peel with array-induced voltage approach [12], pectin extraction from walnut processing waste with ultrasound-assist, high-viscosity apple peel pectin extraction with citric acid [13], pectin and phenolic compounds from sour cherry pomace (SCP) with microwave-assisted approach [9] and also pectin extraction from orange peel by a combination of surfactant and microwave-assisted extraction methods [14]. Especially pectin extraction from the apple pomace is easily processible and safely utilized in industrial processes and also eco-friendly method [3, 4].

The effects of dietary pectin or modified citrus pectin have been studied in the literature and it was concluded that pectin rich diet can reduce blood cholesterol, supports immune system and decreases tumorigenic activity [15-17]. In a study, increased number of surviving stem cells in intestine of mice which were fed with pectin rich diet and exposed to total-body gamma-IR was showed. This result indicates a radioprotective property of dietary citrus pectin [18]. Pectin extracted from cacao pod husks after deacetylation and de-esterification processes provides antitumoral activity [19]. The anti-tumorigenic activity of modified citrus pectin is generally related with its galectin-3 antagonism which is a member of lectin family and plays an important role in cancer and development of fibrotic disease [20]. Cancer stem cells are an important subgroup of cancer cells in tumor tissues. They exhibit increased resistance to drug molecules and other cancer treatment strategies. Their chemoresistance is mainly because of high expression of multi drug resistance proteins such as ABC transporters [21-23]. In addition, increased expression of galectin-3, is related to cancer development and metastasis, in tumor cells [24, 25] and cancer stem cells are shown in the studies [26]. There are some therapeutic strategies for inhibition of cancer stem cells including monoclonal antibodies and micro RNA technologies [23] and different types of pectin molecules may help the inhibition of cellular activity.

For tissue engineering applications pectin, is generally used in combination with other materials such as chitosan, hydroxyapatite, poly-L-lactone, and gelatin to increase its mechanical properties in the fabrication of 3D hydrogels [27, 28]. Pectin containing injectable biomaterials [29] and 3D matrices which supports cell attachment and proliferation can also be prepared by electrospinning process [30, 31]. In a study, vascular cell differentiation of mesenchymal stem cells (MSCs) was investigated on electrospun pectin hydrogel nanofibers. The cells were able to retain their viability for 14 days on the hydrogel nanofibers and their differentiation was changed with the stiffness of the hydrogels and was triggered in the direction of vascular cells [32].

Human body possesses limited regenerative capacity in organ or tissue repair, e.g. in pathologic conditions or in degenerative diseases. Regenerative medicine field highlights the function of stem cells for tissue repair and regeneration of organs because of their unique properties such as self-renewal, proliferation and differentiation into desired and various cell types [33, 34]. Mesenchymal stem cells are found almost in every tissue in our body such as, dental pulp, craniofacial bone, dental follicle, tooth germ, apical papilla, oral mucosa, gingiva, bone marrow, adipose tissue and periosteum [35-38]. The effects of pectin biomacromolecule on behavior of MSCs have been investigated. In a study, human amniotic membrane MSCs were cultured in hydroxyapatite/LM pectin composite gel structure. It was shown that MSCs preserved their osteogenic differentiation capacity and inhibition of T cell proliferation in the presence of LM pectin, also they did not trigger immune response [39]. Pectin/chitosan membrane can provide attachment, adhesion and proliferation of adipose-derived stem cells (ADSCs) [40]. Moreover, bone shaft

MSCs on chitosan/gelatin/pectin network (CGP) films exhibit high ALP activity, osteogenic gene expression and increased mineral formation capacity due to the suitable physicochemical and mechanical features of CGP network films. These results indicate that the network film has the potential to control the MSCs fate [28]. In another study, the biocompatibility of vancomycin hydrochloride including poly (L-lactide) fibers loaded silk fibroin/oxidized pectin hydrogels was shown by MTT assay. Human adipose tissue derived MSCs preserved their viability for eight day cell culture study [41].

Dental pulp stem cells (DSCs) are post-natal stem cell group which have similar properties to MSC with their self-renewal capacity, high proliferation rate and multilineage differentiation potential [42]. Dental pulp SCs can be isolated from third molars and deciduous teeth (DPSCs) without any invasive surgical process, so that they are a promising group of SC with their ease of obtain. Therefore, DPSCs are valuable and crucial MSC sources for tissue engineering and regenerative medicine studies [43]. Dental pulp stem cells involve neural crest stem cells and they have potential to differentiate into neurogenic, adipogenic, chondrogenic, osteogenic cell types [44, 45]. The studies which include pectin biomolecule and MSC interaction is generally based on bioscaffolds. Pectin as a part of biomaterial or as a soluble agent may trigger different signaling pathways in MSCs. To the best of our knowledge, there is no study in which the effects of the presence of soluble pectin in cell culture medium on human mesenchymal stem cells were investigated. In this study, it was aimed to investigate the effects of dissolved citrus pectin in culture medium on human dental pulp stem cells viability and morphology.

## 2. MATERIALS AND METHODS

### Human Dental Pulp Stem Cells Culture

Human dental pulp stem cells (hDPSCs) which were isolated and characterized in a previous study [39] were a kind gift from Prof. Dr. Menemşe Gümüřdereliođlu, from Hacettepe University, Turkey. The cells were cultured in 15% (v/v) fetal bovine serum (FBS, Biowest, France) including alpha-Minimum Essential Medium ( $\alpha$ -MEM, Biochrom, Germany). The antibiotics were used as [10 units/mL penicillin and 10  $\mu$ g/mL streptomycin (Sigma, Germany), 50  $\mu$ g/mL gentamicin (Sigma, Germany) and 0.25  $\mu$ g/ mL Amphotericin B (Sigma, Germany)] at 37 °C in 5% CO<sub>2</sub> atmosphere (Heraus, Germany). The cells were detached from the surface by trypsinization at 80% confluency and cryopreserved for further studies. Human DPSCs at passage 5 and 6 were used in the studies.

### Pectin Solution Preparation

Stock pectin (Galacturonic acid  $\geq$ 74.0%, Sigma Aldrich) solution was first prepared by dissolving 50 mg citrus pectin in 250  $\mu$ l absolute ethanol [7]. After complete dissolution the mixture was added into 4750  $\mu$ l  $\alpha$ -MEM medium and was filtered through 0.22  $\mu$ m syringe filter. After filtration, corresponding reagents were added to this solution to obtain 15% FBS (v/v), 1% (v/v) L-glutamin and 1% (v/v) antibiotic mixture in the final stock solution with 8.5 mg/mL pectin concentration.

### Cell Viability Assays: Resazurin Application and MTT Assay

Human DPSCs were cultured on 24 well plates as  $10 \times 10^3$  cells/cm<sup>2</sup> in culture medium before application of pectin solution. After 24 hours, the culture media of the cells were changed with the fresh culture medium for cell control group, with pectin including media (0.43, 0.85 and 1.7 mg/mL) for sample groups and ethanol including medium (0.85%, v/v) for negative control group. Pectin concentrations were selected within the range of investigated concentrations according to the literature [7, 46, 47]. The cells were incubated in these media for eight days at 37 °C in 5% CO<sub>2</sub> atmosphere.

Resazurin stock solution (Sigma, Germany) was prepared as 0.8 mM in Ca+2 Mg+2 containing PBS (Biowest, France, PBS+). The solution was filtered through 0.22  $\mu$ m syringe filter and kept at +4o C during

experiments. This stock solution was diluted by culture medium just before the application to the cells. The used medium on the cells were aspirated and fresh resazurin working solution (1/10 dilution in culture medium) was applied to the wells. The color change of the medium was observed after 4 hours at days one and eight. Due to the non-toxicity of resazurin on the cells, the used resazurin containing medium was collected for analysis after application. Cells were washed with PBS+ and fresh positive-negative control and pectin including media were applied to the cells. The cells were examined under inverted microscope (Leica, DM IL Led Fluo, Germany).

MTT analysis was carried out at the end of the culture period, at day 8. MTT working solution was prepared by 10 times dilution of 2.5 mg/mL MTT stock solution (Biofroxx, Germany) with culture medium without serum. Used culture media were aspirated from the wells and 600  $\mu$ L MTT working solutions were applied to the cells. The cells were incubated with MTT working solution for 4 hours at 37 °C in 5% CO<sub>2</sub> atmosphere. At the end of incubation, used MTT solutions were withdrawn and 0.04 M HCl containing isopropanol solution was applied to the wells to dissolve newly formed formazan crystals which are indication of mitochondrial activity of viable cells. The corresponding MTT absorbance values were recorded by UV-Vis Spectrophotometer (Agilent Cary 60) at 570 nm in comparison with 690 nm reference wavelength. Cell viability (%) was expressed in relation to the absorbance value of solvent control cells.

### **Live/Dead Cell Imaging**

To assess the live/dead hDPSCs after pectin solution application, Ethidium Homodimer-1 (0.01 M, Eth-1, Abcam, UK) and Calcein AM (0.01 M, Sigma, Germany) dyes were used. Each dye was added into 1% L-glutamin containing PBS+ in 1/1000 dilution to obtain live/dead cell staining solution. Cells were washed once with PBS+ and live/dead cell staining solution was applied for 30 min at room temperature in dark. At the end of incubation period, staining solution was aspirated and the cells were washed once with PBS+ and visualized in PBS+ under a fluorescence microscope (Leica, DM IL Led Fluo, Germany) immediately.

### **F-actin/Nucleus staining**

To observe the cellular cytoskeleton and cell nucleus, F-actin/nucleus staining was carried out. The cells were washed with PBS once and fixed in 4% formaldehyde (v/v in water) solution for 20 minutes at room temperature. After removal of the fixation solution, the cells were washed with PBS and 0.1% Triton X-100 solution in PBS was applied for 8 min to ensure cell membrane permeability. Dye solution was prepared in PBS containing 1% BSA (Bovine Serum Albumin, PBSA) with 2  $\mu$ L/mL Phalloidin-iFluor 488 Reagent (Anti-Factin antibody, Abcam, UK) and 1  $\mu$ g/mL DAPI (Sigma, Germany). After membrane permeability step, the cells were washed with PBSA and they were incubated in dye solution for 90 minutes at room temperature in dark. The cells were washed with PBSA for two times to remove unbound reagents and glycerol solution was added on the cells. The images of immunostained cells were taken under fluorescent microscope

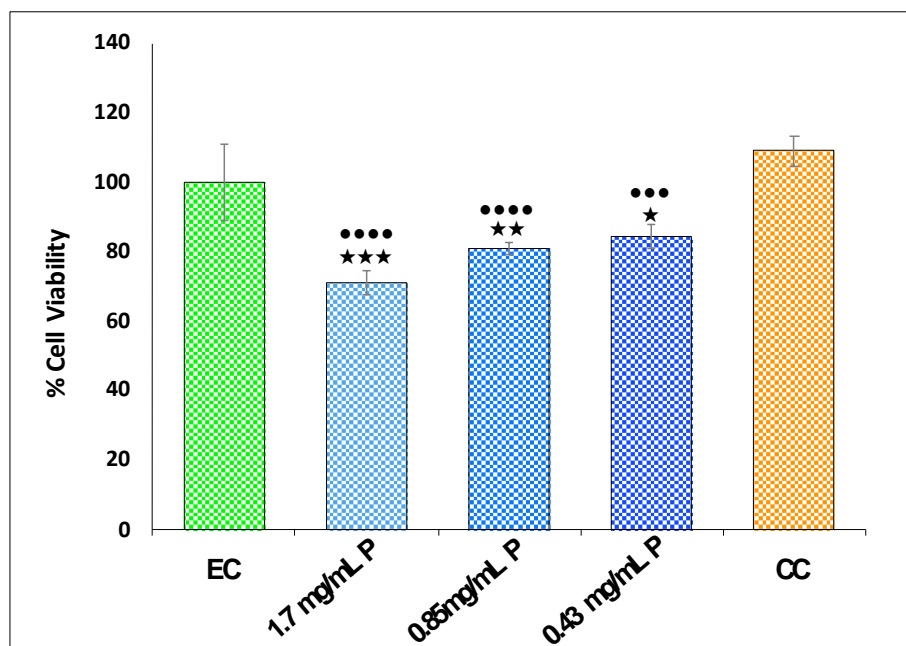
### **Statistical Analysis**

Statistical analysis was performed by one-way ANOVA and Post-hoc Tukey test by GraphPad Prism 8 statistic software program (GraphPad Software, USA).

## **3. RESULTS AND DISCUSSION**

In this study, the effects of dissolved citrus pectin biomacromolecule on human dental pulp stem cells were investigated by means of cell viability and morphological analyses.

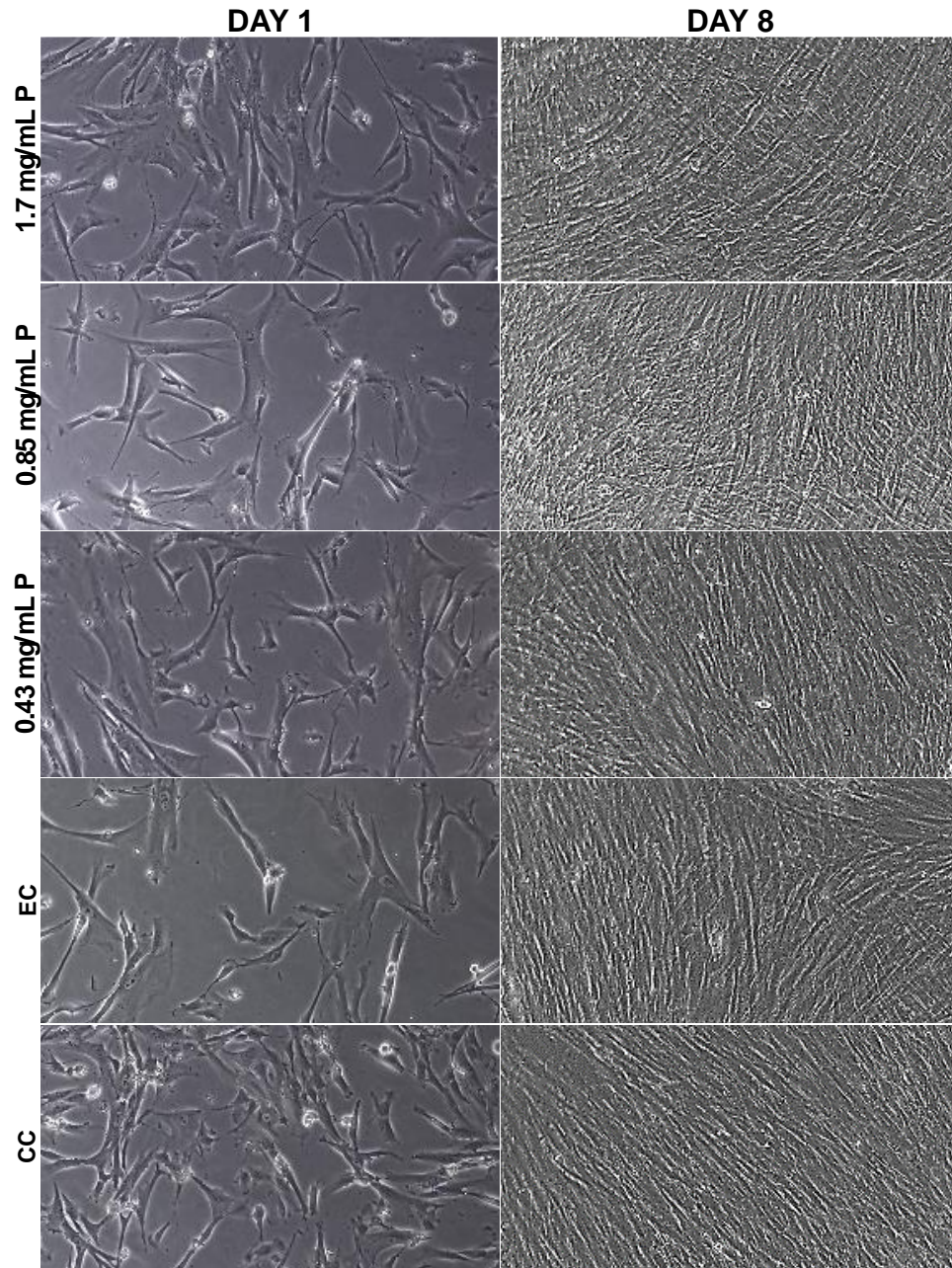
Resazurin sodium salt or resazurin including dye application is widely used for animal, yeast, bacteria and plant cells [48]. It is a non-toxic dye and the cells do not die after application; thus, it allows to track the viability of the same cell group during cell culture studies. The viability of MSCs also has been followed by resazurin dye in our previous studies [49]. In this study, the viability of hDPSCs was investigated by the application of 80  $\mu$ M resazurin sodium salt solution. Mitochondrial activity in both control groups (ethanol group as a solvent control group and cell control group without any reagent application) and pectin applied groups were observed by the color change of culture media from light blue to purple and pink. There was no color difference between the groups on days one and eight according to our visual observations (data not given). Another assay which was carried out for the quantification of viability results of the same cell groups was MTT assay (Fig. 1). It was applied on day 8 and the results given in Fig. 1 were calculated by normalizing the corresponding absorbance values with respect to the absorbance values of solvent control group. It was clearly seen that, dissolved pectin application significantly decreased hDSCs viability when compared to the viabilities of control groups. The most decreased viability was observed in 1.7 mg/mL pectin applied group when compared to the control groups. In a study, the effect of citrus pectin and heat modified citrus pectin (3 mg/mL) in cell culture medium on HepG2 (liver) and A549 (lung) human cancer cell lines was investigated. It was concluded that both pectin types decreased cellular viability and some caspase independent cell death signals were observed in A549 cells [50]. In another study, viability of HaCaT (human keratinocyte) cell line in the presence of high molecular weight citrus pectin and modified citrus pectin with low molecular weight and low degree of esterification was investigated. It was stated that both type of pectin decreased cell viability in the 1-750  $\mu$ g/mL concentration range [51].



**Figure 1.** Viability of hDPSCs (%) by MTT assay on day eight. The absorbance values were normalized with respect to the absorbance values of ethanol applied group. P: Pectin, EC: Ethanol control, CC: Cell control “★” represents the significant difference in comparison with EC and “●” represents the significant difference in comparison with CC ( $p < 0.05$  ★;  $p < 0.01$  ★★;  $p < 0.001$  ★★★, ●●●;  $p < 0.0001$  ●●●●).

Inverted phase contrast images of hDPSCs were given in Fig 2. The morphologies of the cells in every group were as expected from MSCs with thin, bipolar, fibroblastic features (on day 1). After 8-day cell culture, the proliferation of the cells was significantly high, and it was not possible to analyze single cell morphology. However, their high proliferative behavior and growing capacity in multilayers were clearly observed in every group. As a result, there was not any significant change in the cell morphology which

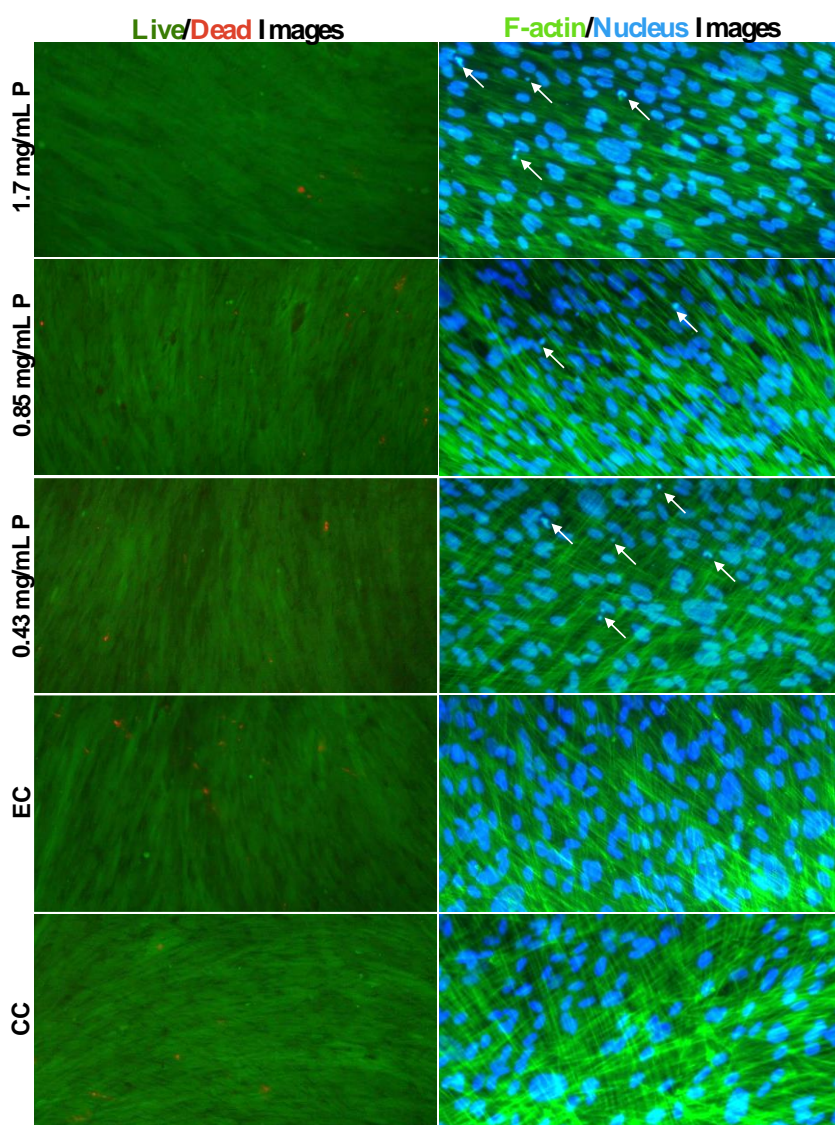
can be related to decreasing cellular activity. Calcein-AM and Ethidium homodimer-1 staining allows visualization of live and death cells at the same time in live cell culture (Fig. 3). Non-fluorescent calcein-AM is a healthy cell membrane permeable dye and turns into fluorescent calcein after intracellular esterase activity. Eth-1 is a nucleic acid binding reagent which allows visualization of cells with membrane damage (necrotic cells) shown in red color. Most of the cells were alive staining in green color and there were a few red points indicating death cells in the culture. Hence, decreasing mitochondrial activity may be a result of suppressed proliferation instead of activated necrotic cell death.



**Figure 2.** Phase contrast images (20X) of hDPSCs on days 1 and 8 before resazurin application under inverted phase contrast microscope. P: Pectin, EC: Ethanol control, CC: Cell control.

Mesenchymal stem cells spread on the surface and organize their filamentous actin stress fibers. In this study, F-actin/nucleus staining showed that hDPSCs fibrous actin filaments were organized in all groups, however, the difference in the morphology of the nuclei of certain cells in the pectin applied groups

was shown by arrows in Fig. 3. Condensed and crescent nuclei was shown in the cells which can be a sign of early stages of apoptotic cell death [52]. Induction of apoptotic pathways in cancer cells in the presence of different types of pectin has been discussed in many studies [53-55]. In a study, autophagy was also observed in A549 cells after heat-modified citrus pectin application in culture medium [50]. Hence, the crescent nuclear shape in hDPSCs of this study may be a sign of apoptotic cell death induced by the presence of pectin in culture medium which can be further validated by apoptotic markers and molecular studies. The effects of the presence of pectin in the 0.1 – 10 mg/mL concentration range were investigated in cancer cells related research in the literature [7, 46, 47]. In this study, 0.43 - 1.7 mg/mL pectin concentration range was selected in order to be comparable with the results in literature studies. However, higher pectin concentrations, longer cell culture periods and different types of pectin molecules should be investigated to observe more significant and specific changes in the viability, morphology and molecular signaling pathways of hDPSCs.



**Figure 3.** Fluorescence images of hDPSC on day eight. Live/dead cell analysis was performed at the end of culture using Calcein-AM for living cells and Ethidium homodimer-1 (Eth-1) for dead cells (10X). Calcein-AM incorporated living cells stained green color and Eth-1 permeable dead cells stained in red color. F-actin and nucleus staining was performed after fixation to analyze cell morphology (20X). Green color shows iFluor 488 conjugated anti F-actin antibody stained filamentous actin fibers, while blue color shows DAPI stained cell nucleus. White arrows show condensed and crescent cell nuclei. P: Pectin, EC: Ethanol control, CC: Cell control.

In this study, the decrease in the viability of hDPSCs may be explained by the interaction of pectin and galectin-3. Galectin-3 as a sub-group of carbohydrate binding galectin family is responsible for cell adhesion, proliferation, survival, tumor cell proliferation and metastasis [25]. It is also expressed in MSCs and can affect immunomodulatory properties of these cells [56]. The inhibition of galectin-3 related signaling pathways such as cell adhesion, survival and proliferation in the presence of different types of pectin were shown in the studies [53, 57]. Galectin-3 binding activity of pH modified citrus pectin was also clearly shown by enzyme linked immunosorbent assay [53] and suitability of some types of assays were studied [58]. Galectin-3 expression in cancer stem cells was also shown in a study [26] but it is a new research area and there are many points waiting to be clarified on this subject. Investigation of the interactions between cancer stem cells, galectin-3 and different types of pectin molecules will contribute to the development of alternative treatment strategies for cancer disease.

#### 4. CONCLUSIONS

In this study, the effects of citrus pectin on human dental pulp stem cell viability and morphology were investigated. It was observed that proliferation of hDPSCs significantly decreased due to the presence of pectin in the cell culture medium for the selected concentration range. In addition, the shape of the nuclei of pectin applied cells showed crescent morphology when compared to the ellipsoid and smooth shape of nuclei in the control cells. This crescent morphology of nucleus may be a sign of apoptotic cell death induction by the presence of citrus pectin. It can be concluded that pectin biomacromolecule can significantly affect the viability and morphology of hDPSCs. Based on this information, it can be suggested that the evaluation of the effects of the pectin molecule on cancer stem cells may also be important.

#### 5. ACKNOWLEDGEMENT

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