Original article

MedInform

DOI: 10.18044/MEDINFORM.2025124.2218

ISSUE 4, 2025

# Isolation and cultivation of dental pulp stem cells from permanent teeth and verification of their identity

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## Abstract

Technologies for tissue engineering are applied for the regeneration of dental pulp. They are based on the use of suitable and accessible cellular sources such as mesenchymal stem cells of dental origin. The creation of cell cultures from stem cells from the dental pulp of permanent teeth and their characterization as such is an important part of carrying out studies on different biomaterials derived from autologous blood.

The aim of the present article is the isolation and cultivation of dental pulp stem cells from permanent teeth and verification of the identity of the obtained cell cultures by immunofluorescent staining. Materials and Methods. Dental pulp was removed from freshly extracted healthy human teeth. By a laboratory protocol, cell cultures of dental pulp stem cells were obtained. They were examined by immunofluorescent staining and analysis of the expression of specific mesenchymal, pluripotent, odontogenic and epithelial markers.

Results. The cells observed under an inverted phase-contrast microscope and the data obtained from the staining demonstrate the mesenchymal origin of the cell cultures.

Conclusion. The results of the present study confirm that, according to the described methodology, the created cell cultures from stem cells are mesenchymal stem cells with odontogenic potential. The application of this methodology in scientific research provides a basis for studying the qualities of autologous blood-derived biomaterials for regenerative restoration of dental pulp affected by an inflammatory process.

***Keywords:*** dental pulp stem cells, mesenchymal stem cells, immunofluorescence, regenerative endodontics, dental pulp, odontogenic differentiation

## *Introduction*

Technologies for tissue engineering are applied for the regeneration of dental pulp. They rely on the use of suitable and accessible cellular sources such as mesenchymal stem cells (MSCs) of dental origin, the application of natural and synthetic biomaterials, the use of growth factors, and combinations of these elements (1). Tissue engineering involves stem cells, fibrin membranes and bioactive growth factors, which form different types of biomaterials. Stem cells are important for the regeneration of tissues after injury. Biomaterials provide the three-dimensional scaffold (matrix) for cell growth and regulate cellular function at the site of application. Growth factors enhance the regenerative effect and regulatory function of stem cells (1).

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Through the methods of regenerative medicine, the structure and function of damaged biological systems are restored. This field of medical science has applications in cardiology, neurology, orthopedics, dentistry and many other specialties (2). As early as 1932 the dentist G. L. Feldman provided evidence for regeneration of the dental pulp under certain biological conditions (3).
Stem cells are one of the pillars of regenerative medicine. They are unspecialized cells with two essential characteristics: they are capable of self-renewal and can differentiate into different cell types. They are found both in embryonic and in mature tissues, and their differentiation potential depends on the stage of development and the cell type (4).
Populations of stem cells can be isolated from different dental structures and from different stages of tooth development. About eight unique populations of mesenchymal stem cells have been isolated and characterized. Postnatal stem cells from the dental pulp of permanent teeth are the first identified stem cells (5). Other stem cells isolated from dental structures include mesenchymal stem cells isolated from the pulp of deciduous teeth (5,6), stem cells of the periodontal ligament (7), progenitor cells of the dental follicle (8), mesenchymal stem cells obtained from alveolar bone (9), stem cells from the apical papilla of human teeth (10), tooth-germ progenitor cells (11) and gingival mesenchymal stem cells (12).
The objective of the present study is the isolation and cultivation of human dental pulp stem cells (hDPSCs) from permanent teeth and the characterization of the obtained cell cultures by immunofluorescent staining.

## *Materials and Methods*

### Isolation and cultivation of hDPSCs

In accordance with the Helsinki Declaration II all subjects provided informed written consent to participate in the study. The Research Ethics Committee of the Medical University of Sofia approved this project (approval number: 14/12.07.2024) as part of the framework for scientific developments and projects involving human research.
Isolation and cultivation of a cell culture from the dental pulp of permanent teeth. Third molars and teeth extracted for orthodontic or surgical indications are used. Only teeth without carious lesions, restorations or periodontal changes that could compromise the integrity of the pulp are included.
After extraction, the teeth are rinsed three times with saline and placed in sterile transport medium DMEM (Dulbecco's Modified Eagle Medium, high glucose, Sigma-Aldrich) with added penicillin 100 U/ml and streptomycin 100 μg/ml. They are transported to the laboratory within 1–2 hours at room temperature.
The extracted teeth are horizontally separated at the cementoenamel junction using a conical diamond bur with constant water cooling until reaching the pulp chamber, ensuring that the pulp is not damaged. The coronal part is carefully separated with an elevator. The pulp tissue is extirpated with fine sterile endodontic instruments or a nerve extractor, avoiding mechanical crushing.
The extirpated pulp is transferred to a sterile Petri dish, cut into fragments (~1 $mm^3$) with a scalpel and transferred into 15ml conical tubes. The fragments are incubated in a enzymatic digestion

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solution: Collagenase type I and Dispase for 60 minutes at 37 °C, 5% $CO_2$, with periodic agitation. After incubation the suspension is centrifuged at 2700 rpm for 3 minutes, the supernatant with enzymes is removed, and the cell pellet is resuspended in a small volume of DMEM.

The cells are cultured in Petri dishes with medium DMEM, 10% FBS (Fetal Bovine Serum, qualified, heat-inactivated; Sigma-Aldrich) and 1% penicillin/streptomycin. Cultivation is carried out at 37 °C, 5% $CO_2$ and saturated humidity. The medium is changed every other day. Upon reaching approximately 80% confluence the cells are passaged into a culture vessel with fourfold larger volume.

The Petri dish with adherent cells is rinsed with PBS (Phosphate Buffered Saline, pH 7.4; Sigma-Aldrich) to remove residual medium and bovine serum. A trypsin solution (10×) is added to detach the cells from the bottom of the culture vessel and the cells are left in the incubator at 37 °C, 5% $CO_2$ for 10 minutes. The culture vessel is then rinsed three times with PBS to collect the cells into a 50-ml tube. This suspension is centrifuged for 3 minutes at 2700 rpm. The supernatant is removed and the pelleted cells are resuspended in DMEM. The cells are transferred into a new culture vessel (flask) with four times larger volume. Each transfer of cells to a new vessel is called a passage and is denoted by a sequential number: P1, P2, P3, etc. Cells with a lower passage number are used for experimental purposes.

**Characterization of stem cells isolated from the dental pulp of permanent teeth by immunohistochemical method using immunofluorescent staining and analysis of the expression of specific mesenchymal, pluripotent, odontogenic and epithelial markers.**

Characterization of the isolated dental pulp cells is a key step to confirm their stem-cell origin. For this purpose immunofluorescent staining is applied, which allows direct visualization of the expression of specific markers. The panel of antibodies used includes mesenchymal markers: Vimentin (a cytoskeletal protein characteristic of mesenchymal cells); CD44 (HCAM) (a surface receptor associated with adhesion and migration); MCAM (CD146) (a surface marker of mesenchymal and perivascular cells); VCAM (CD106) (a cell adhesion molecule expressed by mesenchymal stem cells); N-cadherin (an adhesion protein involved in cell-to-cell interactions); COL3A1 (type III collagen, an extracellular matrix protein); pluripotency and stem markers: CD117 (c-Kit) (a receptor for stem-cell factor associated with progenitor cells); Nestin (an intermediate filament expressed in undifferentiated and neural-like cells); Nanog (a transcription factor key for pluripotency and self-renewal); OCT3/4; as well as functional/odontogenic markers: Alkaline phosphatase (AP) (an enzyme associated with mineralization activity); DSPP (dentin sialophosphoprotein) (an odontogenic protein characteristic of differentiated odontoblasts). As controls epithelial markers that are not expressed by mesenchymal stem cells are included: CK19 (cytokeratin 19) (an epithelial marker absent in MSCs); E-cadherin (an epithelial adhesion protein absent in MSCs).

For immunofluorescent staining, hDPSCs at passage P2, which were isolated and seeded in 24-well plates, are used. The cells are cultured in standard DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin in an incubator at 37 °C, 5% $CO_2$ and saturated humidity until appropriate confluence is reached.

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The cells are washed three times with PBS (pH 7.4) to remove residual culture medium and serum proteins. They are then fixed with 4% formalin (300–500 µl per well) for 15 minutes at room temperature, followed by triple washing with PBS. For permeabilization of cell membranes, 0.05% Triton X-100 (Sigma-Aldrich) in PBS is used, and the cells are incubated for 10 minutes at room temperature. To block nonspecific binding sites the cells are incubated with PBS containing 0.1% BSA (Bovine Serum Albumin; Sigma-Aldrich) for 30 minutes at room temperature. This step reduces background signal and increases the specificity of staining.

**Primary antibodies**

After blocking, the cells are incubated with primary antibodies - 30 µl per well for 1 hour at 4 °C. Antibodies against markers of mesenchymal, pluripotent, odontogenic and epithelial cells were used.

After triple washing with PBS the cells are incubated with the corresponding secondary antibodies diluted 1:100 in PBS/BSA: goat anti-mouse IgG (H+L), conjugated with Alexa Fluor 488 or 594, for primary antibodies of mouse origin, and goat anti-rabbit IgG (H+L), conjugated with Alexa Fluor 488 or 594, for primary antibodies of rabbit origin.

For staining and visualization of cell nuclei after the final triple wash with PBS, DAPI (D9542) - a nuclear fluorescent dye (Sigma-Aldrich) - is added: PBS 300 µl and DAPI 30 µl in each well for 5 minutes. The preparations are observed and documented with an IN Cell Analyzer 6000 at ×20 and ×60 magnification with appropriate filter sets for DAPI and the fluorophores used.

## *Results*

The results on days 10 - 15, recorded with an inverted phase-contrast microscope, are shown in Figure 1.

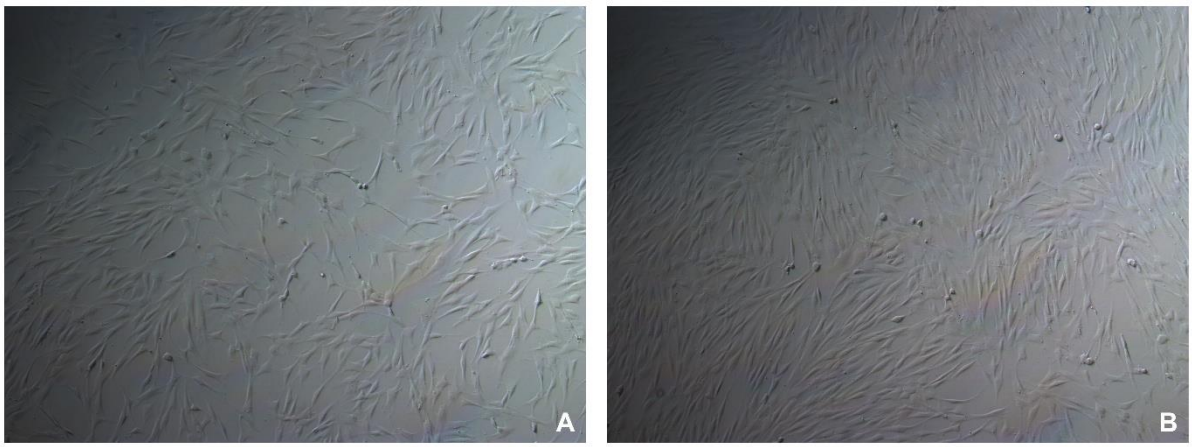


**Figure 1. Morphology of dental pulp stem cells (hDPSCs), images acquired using an inverted phase-contrast microscope (Leica DMI300 B, objective 5x/0.12). (A): cells with fibroblast-like morphology that have adhered. (B) Formed colonies that gradually merge.**

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Morphologically, these cells demonstrate a typical mesenchymal phenotype - an elongated spindle-shaped form with clearly expressed cellular processes. In some fields, heterogeneity is observed: part of the cells have shorter, stellate processes, and others are more elongated, with a parallel arrangement resembling fibroblasts. With increasing confluence, the cells arrange in whorled or parallel-aligned patterns characteristic of mesenchymal cells (Figure 1). The cells divide intensively and over a period of 5 - 10 days gradually form colonies that expand evenly and create a confluent monolayer.

The cells isolated from the dental pulp were subjected to immunofluorescent characterization (IN Cell Analyzer 6000, ×20) with positive and negative markers in order to determine their origin and potential. The results from the characterization of stem cells isolated from the dental pulp of permanent teeth by an immunohistochemical method are shown in Figures 2, 3, 4, 5.

Mesenchymal markers.

There is clearly positive staining for Vimentin with a filamentous cytoplasmic distribution, which confirms the mesenchymal nature of the cells. CD44 (HCAM) shows membranous expression characteristic of adhesion molecules of mesenchymal stem cells. MCAM (CD146) and VCAM (CD106) are also positive, showing membranous and partially cytoplasmic staining consistent with their expression in mesenchymal stem cells. In addition, positive staining for N-cadherin and COL3A1 confirmed the mesenchymal origin and the ability of the cells to synthesize matrix proteins characteristic of connective tissue. The staining can be seen in Figure 2.

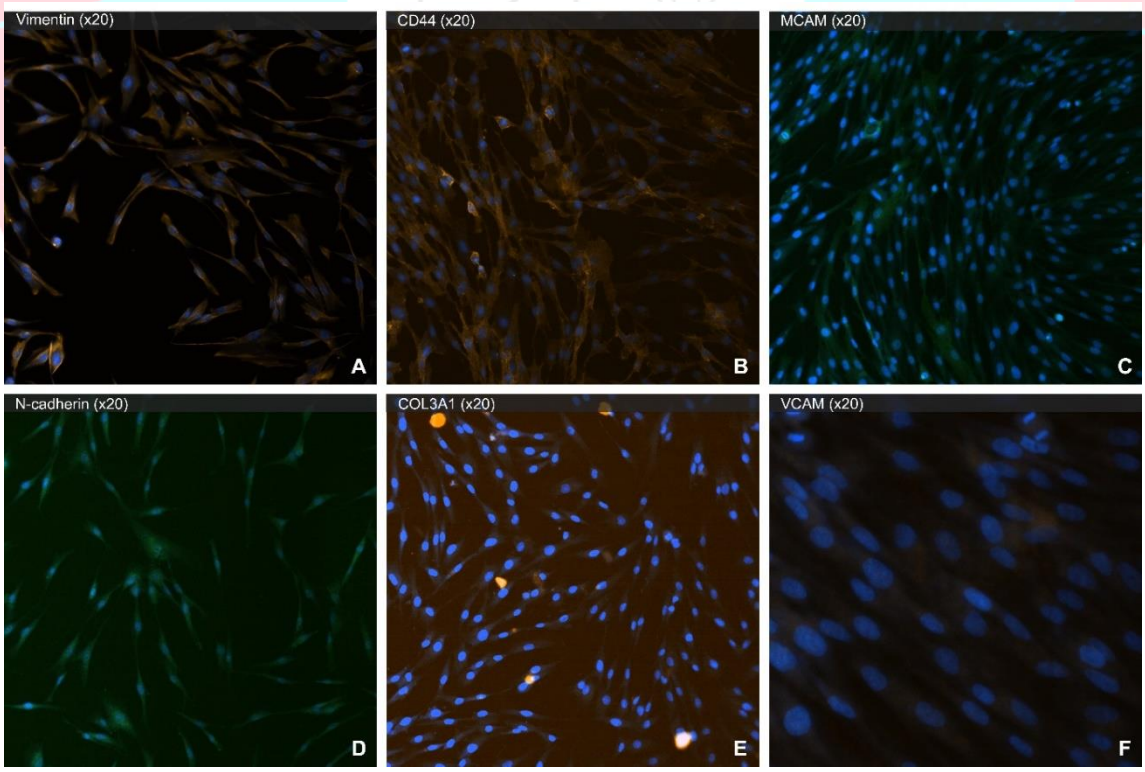


**Figure 2. Immunofluorescent characterization of mesenchymal markers isolated from hDPSCs. The images were acquired with an IN Cell Analyzer 6000 microscope (GE Healthcare) at ×20 magnification. The blue signal (DAPI) shows the nuclei, and**

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**the orange/green fluorescent signal shows the expression of the respective marker: (A) Vimentin - filamentous cytoplasmic staining; (B) CD44 (HCAM) - membranous staining; (C) MCAM (CD146) - membranous/cytoplasmic expression; (D) N-cadherin - membranous staining; (E) COL3A1 - cytoplasmic expression associated with extracellular matrix synthesis; (F) VCAM (CD106 - diffuse cytoplasmic/membranous staining.**

Stem (pluripotency) / early markers.
The cells express Nestin, which is observed in the cytoplasm as filamentous structures. Nanog, located in the nuclei, corresponds to its role as a transcription factor. CD117 (c-Kit) appears as spotted cytoplasmic and perinuclear staining, consistent with its role as a receptor for stem-cell factor. These markers demonstrate preservation of an undifferentiated state and stem-cell potential (Figure 3).

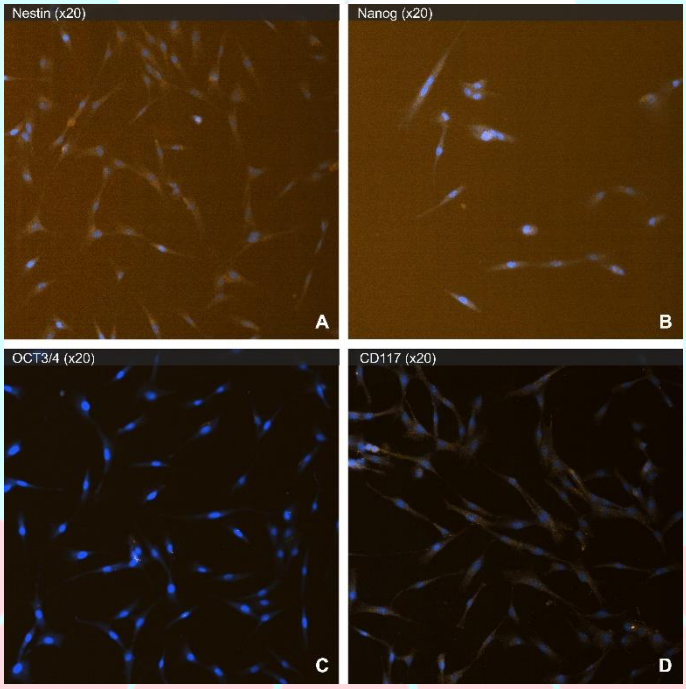


**Figure 3. Stem (pluripotency)/early markers. The images were acquired with an IN Cell Analyzer 6000 microscope (GE Healthcare) at ×20 magnification. (A) Nestin - filamentous cytoplasmic staining; (B) Nanog - nuclear staining; (C) OCT3/4 – nuclear staining; (D) CD117 (c-Kit) - granular cytoplasmic and perinuclear staining.**

Functional and odontogenic markers.
Staining for alkaline phosphatase (AP) is positive with diffuse cytoplasmic distribution, indicating early mineralization activity. DSPP is expressed perinuclearly and granularly in the cytoplasm, corresponding to the secretory function of odontoblasts. This demonstrates the potential of the

DOI: 10.18044/MEDINFORM.2025124.2218 ISSUE 4, 2025

cells to differentiate into odontoblast-like cells and to participate in mineralization processes (Figure 4).

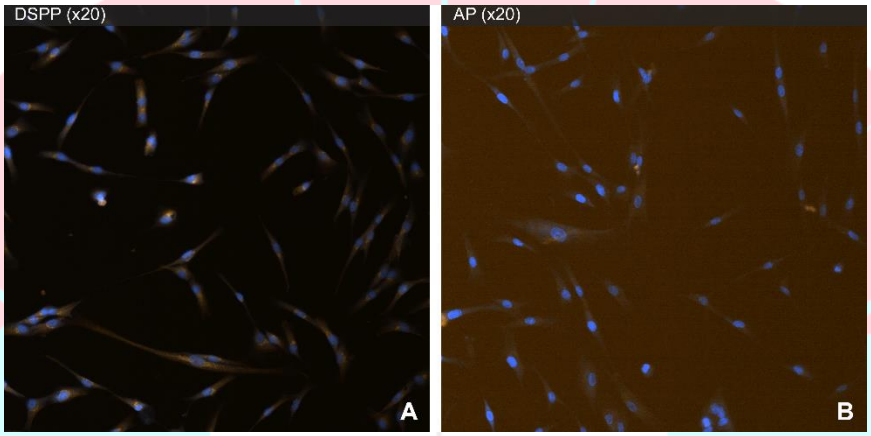


**Figure 4. Functional/odontogenic markers. The images were acquired with an IN Cell Analyzer 6000 microscope (GE Healthcare) at ×20 magnification. (A) DSPP - perinuclear and cytoplasmic expression; (B) Alkaline phosphatase (AP) - diffuse cytoplasmic staining.**

Epithelial (negative) markers.
The cells did not express CK19 and E-cadherin, which suggests the lack of epithelial origin and confirms their mesenchymal nature (Figure 5).

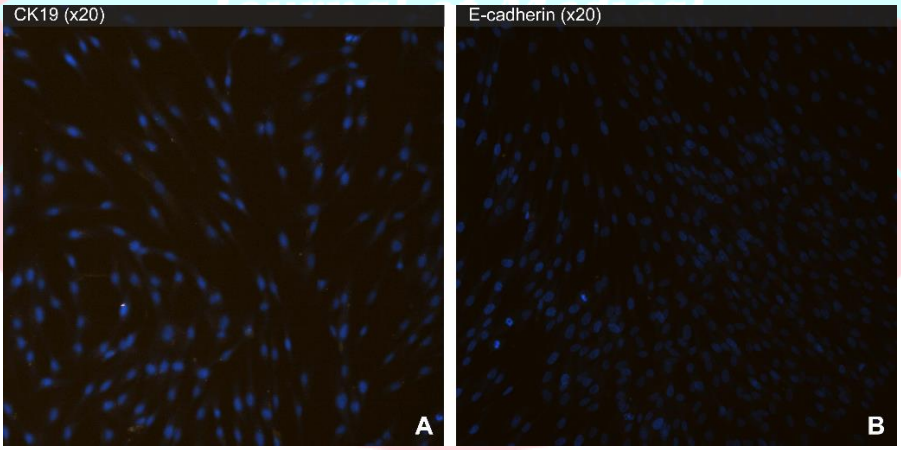


**Figure 5. Epithelial (negative) markers. The images were acquired with an IN Cell Analyzer 6000 microscope (GE Healthcare) at ×20 magnification. (A) CK19 - absent or minimally expressed; (B) E-cadherin - absent or minimally expressed.**

## *Discussion*

The isolation and cultivation of dental pulp stem cell cultures from permanent teeth and their characterization as such is fundamental when investigating the qualities of different biomaterials

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derived from autologous blood. The described methodology for cultivation of hDPSCs and their characterization is an important stage that determines the reliability of various studies. This is confirmed by studies of Miteva et al. (13) and Hirata TM et al. (14). The expression of mesenchymal markers confirmed that the cells isolated from the pulp have the characteristics of mesenchymal stem cells (MSCs). Vimentin was strongly expressed in the cytoplasm of almost all cells, consistent with its established role as a universal mesenchymal marker (5). CD44 showed clearly expressed membranous expression, characteristic of MSCs, corresponding to the observations of Miura et al. (6). CD146 (MCAM) was positive in a significant proportion of cells, which is in agreement with the described perivascular localization of MSCs in the pulp (15,16). VCAM was expressed heterogeneously and weakly, which is consistent with data that this adhesion marker appears in certain subpopulations or under stimulated conditions (17). The combination of these markers confirms that the isolated cells match the immunological profile of hDPSCs. The expression of Nanog and OCT3/4 was recorded in a limited subpopulation of cells, with nuclear and perinuclear localization of the signal. This result indicates heterogeneity of the cell culture and the presence of cells with retained pluripotent potential. Similar mosaic expression has been described in the literature, where Nanog and OCT4 (OCT3/4) are detected at low levels in hDPSCs (16,17). Nestin was also positive, consistent with its role as a marker for undifferentiated cells with neural-like properties. CD117 (c-Kit) was expressed in a limited number of cells, confirming the presence of early stem characteristics. DSPP expression was recorded in the cytoplasm of a subpopulation of cells, which is indicative of odontoblast-like differentiation and corresponds to published data on the role of DSPP in dentinogenesis (18). Alkaline phosphatase (AP) was expressed heterogeneously, consistent with data that this enzyme is an indicator of osteo- and odontogenic differentiation under certain culture conditions (19). COL3A1 showed fibrous and punctate staining corresponding to extracellular matrix production and confirming the activity of DPSCs in the synthesis of structural proteins. CK19 and E-cadherin did not show specific expression, which is expected and corresponds to the lack of epithelial origin in DPSCs. N-cadherin showed weak to moderate expression at intercellular contacts, which is explained by its role in cell adhesion and is not associated with an epithelial phenotype. The data correspond to the observations of Deng et al. (20), who report that N-cadherin can regulate the differentiation of DPSCs but is not an epithelial marker.

The results of the present study confirm that the cell cultures created by the described methodology are mesenchymal stem cells with odontogenic potential. Applying this methodology in scientific research provides a basis for studying the qualities of autologous blood biomaterials for regenerative restoration of dental pulp affected by an inflammatory process. The combination of positive mesenchymal and stem markers confirms that the isolated cells from dental pulp are mesenchymal stem cells with odontogenic potential and that the methodology applied to create this type of cell cultures is reliable as a basis for future studies.

## *Conclusion*

The results of the present study confirm that the cell cultures created by the described methodology are mesenchymal stem cells with odontogenic potential. Applying this methodology in scientific

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research provides a basis for studying the qualities of autologous blood-derived biomaterials for regenerative restoration of dental pulp affected by an inflammatory process.

***ACKNOWLEDGEMENT***

This study is financed by the European Union-NextGenerationEU, through the National Recovery and Resilience Plan of the Republic of Bulgaria, project BG-RRP-2.004-0004-C01 "Strategic research and innovation program for development of Medical University – Sofia"

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Vladova R, Kirilova J, Yancheva S. Isolation and cultivation of dental pulp stem cells from permanent teeth and verification of their identity. J. Med. Dent. Pract, 2025; 12(4):2218-2227.