The effect of ionizing radiation from dental radiographic devices on dental pulp stem cells

Onur Evren Kahraman¹, Tahir Karaman², Tuba Talo Yıldırım³, Ebru Önalan⁴, Serkan Dündar³, Ahmet Tektemur⁴

¹ Firat University, Faculty of Dentistry, Department of Oral and Maxillofacial Surgery, Elazığ, Türkiye
² Firat University, Faculty of Dentistry, Department of Prosthodontics, Elazığ, Türkiye
³ Firat University, Faculty of Dentistry, Department of Periodontology, Elazığ, Türkiye
⁴ Firat University, Faculty of Medicine, Department of Medical Biology, Elazığ, Türkiye

Received: 23 February 2023
Accepted: 30 June 2023

Correspondence:
Dr. Onur Evren KAHRAMAN
Firat University, Faculty of Dentistry, Department of Oral and Maxillofacial Surgery, Elazığ, Türkiye.
E-mail: oekahraman@firat.edu.tr

Abstract

Aim: This study aims to investigate the effects of exposure to different doses delivered by radiographic diagnostic tools on cell death pathways in isolated dental pulp stem cells on a molecular level using qRT-PCR data.

Methodology: Seventy fully impacted mandibular third molars were used for the study. Extracted third molars were divided into seven groups (n = 10 each): A control group receiving no radiation, periapical (PER), panoramic (PAN), and dental tomography (CBCT) groups receiving one dose of radiation each, and periapical, panoramic, and CBCT groups receiving three consecutive doses of radiation each. Then, gene expressions in dental pulp stem cells were evaluated using the qRT-PCR this method.

Results: A significant difference was found in the expression of the ULK1 gene in the dental pulp tissue samples from the PAN1 group; GABARAP, LAMP3, APAF1, BCL2, CASPASE2, and CASPASE7 genes from the PER1 group; the ULK1 gene from the PER3 group; and ATG12, ATG16L1, ATG4A ATG5, BECN1L1, GABARAP, LAMP1, LAMP3, MAP1LC3B, ULK1, APAF1, BCL2, CASPASE2-3, CASPASE-7, and CASPASE-8 genes from the CBCT3 group.

Conclusion: While the radiation doses we applied to the DPSC cell culture are considered low levels, there was an increase in cell death markers with increased exposure to radiation through multiple radiography procedures. Taking measures to prevent errors during radiography and avoiding repeated, unnecessary patient exposure to radiation would help minimize the damaging effects of radiation.

Keywords: Dental pulp stem cells, qRT-PCR, mRNA expression, cell culture, ionizing radiation, radiography
Introduction

Stem cells have a vital role in the repair of all organs and the self-renewal capacity of tissues. Stem cells offer the possibility of developing novel strategies to regenerate missing tissues and treat diseases due to their unique ability to renew themselves and differentiate into various types of cells (1). In dentistry, stem cell research has grown rapidly in recent years, and trials are underway to use stem cells to repair and renew dental structures (2). Stem cells obtained from several tissues are being used for tissue repair (3-5). The primary sources of adult stem cells in oral and maxillofacial regions include the bone marrow (BMSCs), dental pulp (DPSCs), deciduous teeth (SHED), periodontal ligament (PDLCs), dental follicle (DFS), tooth germ (TGP), apical papilla (SCAP), oral epithelium (OES), gingiva (GMCs), periosteum (PSC), and salivary gland (SGSC) (1). Stem cells can be readily harvested from dental pulp tissue and used in research studies without the need for complex and challenging procedures (6).

Extracted teeth are used as the source of stem cells in dental stem cell studies. These teeth include third molars that need to be extracted, deciduous teeth, supernumerary teeth, and teeth extracted for orthodontic purposes (7-9). Stem cells retrieved from the dental pulp are multipotent clonogenic mesenchymal cells with a high capacity for proliferation and a high degree of plasticity (8). The multipotent nature of DPSCs and their effective isolation in recent studies have led to the increased use of these cells in stem cell research (6,10).

Morphologically, cell death occurs via three pathways under physiological conditions: Apoptosis, autophagy, and necrosis (11). Apoptosis is characterized by cell shrinkage, chromatin condensation, nuclear fragmentation, cytoplasmic membrane blebbing, and debris clearance by neighboring phagocytes (12, 13). Autophagy-dependent cell death is differentiated by cytoplasmic vacuolization, formation of autophagosomes, and clearance of materials by lysosomes (12, 14). Necrosis leads to swelling and lysis of the cell, inflammation, and release of intracellular components. It was suggested that these different forms of cell death might be more accurately classified based on molecular characteristics rather than conventional morphological definitions (15).

Radiological imaging modalities have an essential role in diagnosing existing problems of patients presenting to a dental clinic (16). Radiological imaging methods used in dental practice are broadly classified as intraoral and extraoral. The intraoral imaging technique provides perilapical and occlusal views and allows examination of a localized area in two dimensions, whereas extraoral imaging modalities provide panoramic and cephalometric radiographs. Panoramic radiographs are often preferred because, in a single image, they allow good visualization of the maxilla and mandible on the frontal plane with an overall coverage of dental arches. Dental tomography is used for the three-dimensional evaluation of maxillofacial structures. Dental tomography enables the assessment of dental structures in three dimensions and more precisely compared to conventional radiographs (17). Harmful ionizing radiation is released during the use of these tools. It was reported that radiation exposure in humans often occurs due to small doses taken over an extended period rather than a single large dose (18).

The present study aims to investigate the effects of exposure to different doses delivered by radiographic diagnostic tools (periapical, panoramic, and CBCT) commonly used in dental practice on cell death pathways in isolated dental pulp stem cells on a molecular level using qRT-PCR data.

Materials and Methods

Sample retrieval

Prior to the initiation of the study, approval (No. 2016/11-03) was obtained from the Fırat University Ethics Committee for non-interventional procedures, and patients signed an informed consent form for the use of their extracted third molars for research purposes in the study.

Extraction of impacted third molars was performed at the Oral and Maxillofacial Surgery Clinic, Dental Research and Practice Center of Fırat University, and stem cell isolation was conducted at the laboratory of the Department of Medical Biology and Genetics Laboratory, Fırat University School of Medicine.

Seventy fully impacted mandibular third molars were used for the study. Extracted third molars were divided into seven groups (n = 10 each): A control group receiving no radiation; periapical (PER), panoramic (PAN), and dental tomography (CBCT) groups receiving one dose of radiation each; and periapical, panoramic, and CBCT groups receiving three consecutive doses of radiation each.

The study population consisted of patients aged 19 to 25 with an indication for extraction of a fully impacted third molar without any systemic disease. Before tooth extraction, all patients were asked to rinse their mouths with 0.2% chlorhexidine, and the extraction site was wiped with chlorhexidine.

Cell culturing

Following tooth extraction, third molars were stored in a vehicle solution for 2 hours. Periodontal tissues on the teeth were removed by scraping. Extracted third molars were irrigated with isotonic normal saline solution, then again with alcohol for sterilization. Next, the tooth was stored in a medium containing an antibiotic mixture of penicillin and cephalosporin for 1 hour. A deep groove was created to collect dental pulp using an aerator under water cooling, taking the enamel-cement junction as a reference landmark. To expose the pulp chamber, the crown and...
root were compressed with dental forceps to break the tooth, and the pulp chamber was reached through the opened groove. The dental pulp tissue was transferred into a culture medium (Celprogen Inc., Torrance, CA, USA) with the aid of a sterile gripper. The collected tissue was minced into small pieces using a surgical blade prior to enzymatic digestion. The fragments of dental pulp tissue were digested with collagenase type 1 (Gibco ThermoFisher, Waltham, MA, USA) in Alpha-MEM (Capricorn Scientific GmbH, Ebsdorfergarden, Germany) medium containing 10% fetal bovine serum. Following incubation, the solution was agitated gently to isolate cells from tissue fragments and obtain a single cell suspension. The cells from the single cell suspension were loaded into a 6-well plate (Costar Inc., Glendale, AZ, USA) with Alpha-MEM (Capricorn Scientific GmbH, Ebsdorfergarden, Germany) supplemented with 10% fetal bovine serum and cultured in an incubator (Nüve A.Ş., Ankara, Türkiye) in a humidified atmosphere containing 5% CO2.

RNA isolation and exposure to radiation

Stem cell cultures were exposed to 1 radiation dose each of periapical radiography, panoramic radiography, and dental tomography for the first three groups and three consecutive radiation doses of periapical radiography, panoramic radiography, and dental tomography for the other three groups. All devices were set at “average adult individual” mode to simulate routine doses used in clinical practice. The following exposure settings were used for each dose: 6.6 kV, 6 mA, and 0.063 sec for periapical radiography; 68 kV, 5.6 mA, and 16 sec for panoramic radiography, and 90 kV, 10 mA, and 18 sec for CBCT. During panoramic and CBCT radiography, the cells in 25 cm² polypropylene flasks were placed on the bite block of the device. For periapical radiography, the stem cells were placed 15 cm away from the image receptor. The control group did not receive any radiation.

After exposure to radiation, Trizol (Hibrogen, Istanbul, Türkiye) was used to isolate RNA from the stem cells. To obtain RNA pellets, the cells were centrifuged at +4°C at 10,000 g for 20 min and washed with 1 ml of 75% ethanol after discarding the supernatant. Following centrifugation at +4°C at 10,000 g for 5 min, this washing step was repeated to remove the ethanol completely. The pellets were resuspended in 30 μl of nuclease-free water and stored at -80°C until the time of analysis (6). Prior to spectrophotometric RNA quantitation, a blank measurement was performed with sterile water free of DNase/RNAase. One μl of RNA was placed onto the measurement window of a BioSpec-nano (Shimadzu Inc., Kyoto, Japan) device to quantify the RNA yield in ng/μl. This procedure was repeated for each sample.

Complementary DNA (cDNA) Synthesis

RNA samples were used for complementary DNAs (cDNAs) to determine differences in the expression of relevant genes at the mRNA level. A OneScript cDNA synthesis kit (ABM Inc., Richmond, BC, Canada) was used for cDNA synthesis. The samples were placed in a thermal cycler and kept at 25°C for 10 min, 42°C for 50 min, and 85°C for 5 min and at a final temperature of 4°C. Synthesized cDNA samples were stored at -20°C.

Complementary DNA (cDNA) amplification via Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The synthesized cDNAs were amplified via qRT-PCR in the presence of array-specific primers that represent autophagic and apoptotic genes generated by realtimeprimers.com. Beta-actin (ACBT) was used as the reference gene when examining mRNA expression levels. Relative changes in gene expression were analyzed using the 2ΔΔCT method.

One-μl samples of cDNA were placed into each well of 96-well plates for the qRT-PCR analysis, which was performed in triplicate. For each sample, 5 μl of Master Mix (ABM Inc., Richmond, BC, Canada), 0.5 μl of primer (Real Time Primers, LLC, Elkins Park, PA, USA), 0.2 μl of Rox (Invitrogen Inc., Carlsbad, CA, USA), and 3.3 μl of nuclease-free water, calculated according to the number of wells to be used, were prepared in Eppendorf tubes and vortexed. After a pipette was used to add 9 μl of the reaction mixture to the cDNA samples placed in the wells, the plate was covered with optic film.

Statistical analysis

Analyses were performed by using SPSS software (IBM SPSS V24, IBM Inc., Armonk, NY, USA). A statistically significant difference was defined as a p-value less than 0.05.

The independent samples t-test was used to determine differences among the groups in terms of fold increase in mRNA levels. The data for each diagnostic tool were compared to those of the control group.

Results

As can be seen from Table 1, a significant difference was found in the expression of the ULK1 gene in the dental pulp tissue samples from the PAN1 group versus the control group (p<0.05), but there was no significant difference in the expression of other genes between the PAN1 group and the control group (p>0.05). The expressions of genes in the PAN3 group were not significantly different from those in the control group (p>0.05).

A significant difference was found in the expressions of the GABARAP, LAMP3, APAF1, BCL2, CASPASE2, and CASPASE7 genes in the dental pulp tissue samples from the PER1 group in comparison to the control group (p<0.05), whereas no significant differences were observed in the expressions of other genes from the PER1 group versus the control group (p>0.05).
Table 1. Changes in mRNA expressions of autophagic and apoptotic genes according to groups as a result of ionizing radiation to dental pulp tissue samples

<table>
<thead>
<tr>
<th>Gene name</th>
<th>PAN1 mRNA fold increase</th>
<th>PAN3 mRNA fold increase</th>
<th>PER1 mRNA fold increase</th>
<th>PER3 mRNA fold increase</th>
<th>CBCT1 mRNA fold increase</th>
<th>CBCT3 mRNA fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATG12</td>
<td>0.82</td>
<td>0.43131</td>
<td>1.55</td>
<td>0.1401</td>
<td>1.87</td>
<td>0.063821</td>
</tr>
<tr>
<td>ATG16L1</td>
<td>0.62</td>
<td>0.11572</td>
<td>0.88</td>
<td>0.592792</td>
<td>1.4</td>
<td>0.222114</td>
</tr>
<tr>
<td>ATG2A</td>
<td>1.09</td>
<td>0.72254</td>
<td>1.28</td>
<td>0.345923</td>
<td>1</td>
<td>0.991542</td>
</tr>
<tr>
<td>ATG3</td>
<td>0.79</td>
<td>0.36577</td>
<td>0.77</td>
<td>0.331062</td>
<td>0.66</td>
<td>0.15654</td>
</tr>
<tr>
<td>ATG4A</td>
<td>1.13</td>
<td>0.26035</td>
<td>1.57</td>
<td>0.131504</td>
<td>1.8</td>
<td>0.054738</td>
</tr>
<tr>
<td>ATG5</td>
<td>1.15</td>
<td>0.26592</td>
<td>1.16</td>
<td>0.567849</td>
<td>1.16</td>
<td>0.55019</td>
</tr>
<tr>
<td>BECN1L1</td>
<td>0.99</td>
<td>0.26681</td>
<td>1.01</td>
<td>0.985835</td>
<td>1.21</td>
<td>0.452806</td>
</tr>
<tr>
<td>GABARAP</td>
<td>1.73</td>
<td>0.26143</td>
<td>1.84</td>
<td>0.067289</td>
<td>3.58</td>
<td>0.012095</td>
</tr>
<tr>
<td>ATG9A</td>
<td>1.03</td>
<td>0.91181</td>
<td>1.07</td>
<td>0.785832</td>
<td>1.09</td>
<td>0.74337</td>
</tr>
<tr>
<td>LAM1P1</td>
<td>0.74</td>
<td>0.26234</td>
<td>1.87</td>
<td>0.063821</td>
<td>1.59</td>
<td>0.123527</td>
</tr>
<tr>
<td>LAM3P3</td>
<td>0.94</td>
<td>0.13593</td>
<td>1.8</td>
<td>0.072966</td>
<td>2.51</td>
<td>0.024943</td>
</tr>
<tr>
<td>MAP1LC3A</td>
<td>1.39</td>
<td>0.33678</td>
<td>1.15</td>
<td>0.585904</td>
<td>1.47</td>
<td>0.175797</td>
</tr>
<tr>
<td>MAP1LC3B</td>
<td>0.86</td>
<td>0.55725</td>
<td>0.82</td>
<td>0.431309</td>
<td>1.78</td>
<td>0.077098</td>
</tr>
<tr>
<td>MAP1LC3C</td>
<td>0.7</td>
<td>0.28048</td>
<td>0.85</td>
<td>0.506953</td>
<td>1.47</td>
<td>0.175797</td>
</tr>
<tr>
<td>ULK1</td>
<td>213</td>
<td>0.4029*</td>
<td>1.58</td>
<td>0.127441</td>
<td>1.16</td>
<td>0.567849</td>
</tr>
<tr>
<td>APAF1</td>
<td>1.25</td>
<td>0.39615</td>
<td>1.51</td>
<td>0.159348</td>
<td>213</td>
<td>0.040286*</td>
</tr>
<tr>
<td>BAK1</td>
<td>0.85</td>
<td>0.52331</td>
<td>0.82</td>
<td>0.431309</td>
<td>0.93</td>
<td>0.749565</td>
</tr>
<tr>
<td>BAX</td>
<td>1.04</td>
<td>0.89571</td>
<td>1.14</td>
<td>0.604347</td>
<td>1.29</td>
<td>0.334334</td>
</tr>
<tr>
<td>BCL2</td>
<td>0.95</td>
<td>0.83516</td>
<td>1.07</td>
<td>0.785832</td>
<td>217</td>
<td>0.038419*</td>
</tr>
<tr>
<td>CASPASE2</td>
<td>0.91</td>
<td>0.70587</td>
<td>0.96</td>
<td>0.85708</td>
<td>228</td>
<td>0.032658*</td>
</tr>
<tr>
<td>CASPASE3</td>
<td>0.75</td>
<td>0.28998</td>
<td>1.03</td>
<td>0.918132</td>
<td>1.96</td>
<td>0.053959</td>
</tr>
<tr>
<td>CASPASE7</td>
<td>0.96</td>
<td>0.85711</td>
<td>1.21</td>
<td>0.452806</td>
<td>222</td>
<td>0.035738*</td>
</tr>
<tr>
<td>CASPASE8</td>
<td>0.86</td>
<td>0.55725</td>
<td>0.64</td>
<td>0.134272</td>
<td>0.95</td>
<td>0.813412</td>
</tr>
</tbody>
</table>

* The table shows the gene expression change levels in other groups compared to the control group. p<0.05 was considered significant. (PAN1- single dose panoramic radiography, PAN3- three-dose panoramic radiography, PER1- single dose periapical radiography, PER3- three-dose periapical radiography, CBCT1- single dose CBCT, CBCT3- three-dose CBCT).
There was a significant difference in the expression of the ULK1 gene in the PER3 group compared to the control group ($p < 0.05$), but the expressions of other genes in the PER3 group were not significantly different from those in the control group ($p > 0.05$). Relative to the control group, the expressions of genes in the CBCT1 group were not significantly different ($p > 0.05$).

In the dental pulp tissue samples from the CBCT3 group, the expressions of genes were significantly different in comparison to the control group ($p < 0.05$), but no significant difference was found in the expressions of other genes in the CBCT3 group compared to the control group ($p > 0.05$) (Fig. 1).

**Figure 1.** Clustergram analysis of changes in mRNA expressions of autophagic and apoptotic genes according to groups as a result of ionizing radiation to dental pulp tissue samples.

**Discussion**

Cell death occurs through multiple pathways. Morphologically, three pathways of cell death have been identified: apoptosis, autophagy, and necrosis (12). Necrosis involves the swelling and lysis of the cell, inflammation, and the release of intracellular components (14, 19). Apoptosis is characterized by cell shrinkage, chromatin condensation, nuclear fragmentation, cytoplasmic membrane blebbing, and the clearance of debris by neighboring phagocytes (12, 13). Autophagy-dependent cell death is differentiated by cytoplasmic vacuolization, the formation of autophagosomes, and the clearance of materials by lysosomes (12, 14). In the present study, the primers of the genes associated with autophagy and apoptosis have been used to assess their mRNA expressions.

In the current study, in which cell cultures from dental tissues were exposed to various levels of ionizing radiation, statistically significant changes were observed in the mRNA expressions of autophagic and apoptotic genes, including BECLIN1, BECN1L1, RAPTOR, CASPASE-7, CASPASE-8, TP53, CASPASE-3, and LAMP1. The BECLIN-1, RAPTOR, and CASPASE-8 genes showed increased expression in cell culture samples undergoing both panoramic and periapical radiography, whereas the mRNA expressions of the other aforementioned genes were only increased in samples exposed to periapical radiography. Among these genes, CASPASE-7, CASPASE-8, TP53, and CASPASE-3 were involved in apoptosis, and BECLIN-1, BECN1L1, RAPTOR, and LAMP1 were associated with autophagy (11, 20-22).

Caspases belong to a family of cysteine proteases with 15 members. Their primary role is to mediate programmed cell death (23). One of the members of this family, CASPASE-3, is the executioner of apoptosis. Upon activation by initiator caspases (CASPASE-8 and -9), executioner caspases (CASPASE-3, -6, and -7) lead to apoptosis by cleaving critical proteins for dismantling a cell (24). Several anticancer therapies, cytotoxic drugs, radiotherapy, and immunotherapy aim to destroy tumors by targeting cell death by activating CASPASE-3. Yet recent studies have shown a different aspect of CASPASE-
3. One study demonstrated that instead of acting as a tumor suppressor, CASPASE-3 promotes carcinogenesis following the exposure of the cell to chemicals or radiation (25). Other studies involving human subjects have found improved survival among patients with low CASPASE-3 levels (26).

In the current study, increased expressions of all apoptotic cell death markers were observed in samples from dental pulp stem cell cultures exposed to periapical radiography. Irreversible cell death might have been triggered in these groups. This cell death may be explained by the direct exposure of DPSCs to radiation via cone in periapical radiography groups. In contrast, cell culture from the panoramic radiography group was less affected despite higher radiation doses.

Autophagy is a tightly regulated catabolic process in which intracellular components are degraded by lysosomes during cell growth, development, and tumorigenesis (27). Autophagy has a bidirectional effect on tumor formation. In the tumor microenvironment, autophagy serves as a temporary survival mechanism in response to metabolic stress. However, as cellular stress induces sustained or progressive autophagy, cell death will eventually occur (28). Therefore, the appearance of autophagic genes may not necessarily mean that the cell is destined to die. In our study, a significant increase in the mRNA expression of BECLIN-1 over that of the control group was observed. BECLIN-1 recruits proteins from the cytoplasm for degradation by autophagy or provides membrane components for the autophagic pathway. The inactivation of BECLIN-1 results in an increase in tumor incidence in mice (29). Thus, it was suggested that the BECLIN-1 gene acts as a tumor suppressor (30).

Another autophagy-related gene, LAMP-1, is among the major components of lysosomes. These membrane proteins play a critical role in the formation of autophagosomes. In our study, the coexpression of autophagic genes in the groups undergoing panoramic and periapical radiography may indicate the attempt of the damaged cell to survive.

The molecular mechanisms underlying these different cell death pathways are complex, and recent evidence suggests that there are intricate relationships among them. This intricacy makes it difficult to clearly differentiate one cell death pathway from another (15). The mechanism that drives cell death is often specific to the cell type and intracellular contents, stimuli, and environment. Therefore, we believe it is inappropriate to consider gene expressions related to apoptotic and autophagic cell death as separate entities. A cell in which autophagy has begun due to the formation of autophagosomes will not necessarily die, nor will it necessarily attempt to survive. Autophagic and apoptotic cell death may also occur simultaneously in similar conditions (31,32). Given these data, more cell deaths were observed in the cell culture exposed to three consecutive radiography doses than in the group exposed to only one dose of radiography and the control group in the present study. However, it remains hypothetical whether autophagy is for survival or cell death.

Direct or indirect DNA damage caused by ionizing radiation may impair cell vitality (10). The analyses of mRNA expression are essential for stem cell studies, but not every mRNA results in a particular protein used for the intended purpose. Thus, additional analytical methods, such as Western blot, demonstrate the presence of the protein in question. One of the limitations of our study involved using only mRNA expression analysis. In the current study, dental pulp stem cells were cultured and exposed to direct ionizing radiation. When used at the clinic for diagnostic purposes, however, the beam reaches the cell by passing through soft and hard tissues during exposure to panoramic or periapical radiography. Nevertheless, we believe that our methodology is suitable for demonstrating dose-dependent changes in cell vitality.

**Conclusion**

DPSCs play a critical role in tooth development and regeneration after trauma. While the radiation doses that we applied to the DPSC cell culture were low, there was an increase in cell death markers with increased exposure to radiation through multiple radiography procedures. Radiographic imaging holds an important place in clinical practice in dentistry.

Taking measures to prevent errors during radiography and avoiding repeated, unnecessary patient exposure to radiation would help minimize the damaging effects of radiation.

**Disclosures**

**Ethical Approval:** Ethics committee approval was received for this study from the Fırat University Ethics Committee, in accordance with the World Medical Association Declaration of Helsinki, with the approval number: 2016/11-03.

**Peer-review:** Externally peer-reviewed.


**Conflict of Interest:** No conflict of interest was declared by the authors.

**Funding:** This study was supported by Fırat University Scientific Research Project Foundation Department with the project number DHF.16.04.
References


