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**Review /** Derleme

# Bioactivity Assessment of Pulp Capping Biomaterials: in vitro Approaches

## Pulpa Kaplama Biyomateryallerinin Biyoaktivitelerinin Değerlendirilmesi: in vitro Yaklaşımlar

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**ABSTRACT**

Maintaining the health of the dental pulp is essential for the long-term well-being of a tooth. Vital pulpal therapy is aimed at preserving and supporting the vitality of pulp tissue in teeth affected by trauma, caries, or dental procedures. The primary objective is to stimulate the formation of reparative dentin to maintain tooth viability. Successful outcomes in vital pulpal therapy rely on accurate diagnosis, patient selection, and evaluation of various factors such as pulp exposure location, pulp maturity, and quality of existing dental treatment. In vital pulpal therapy, a protective bioagent, called a pulp capping material, is applied over the exposed dentin. Over time, numerous dental biomaterials have been developed to promote safe tissue responses and improve treatment outcomes. With advancements in our understanding of dentin-pulp complex healing mechanisms, new biomaterials have emerged to support pulp vitality during conservative and restorative dental procedures. However, selecting the most suitable biomaterial for each clinical scenario can be challenging due to the array of options available. Therefore, developing and assessing the bioactivity of novel pulp capping biomaterials is crucial, given the significant role of pulp capping in maintaining dental health. In this review, we review the typical in vitro cell culture and molecular biology techniques frequently utilised to evaluate both the cytoprotective and cytotoxic properties of novel pulp capping biomaterials. These techniques contribute to the comprehensive assessment of biomaterial efficacy and safety, aiding in the advancement of vital pulpal therapy practices and patient care.

**Keywords:** Pulp capping biomaterials, Bioactivity, Biocompability, Cell culture

**ÖZ**

Diş pulpasının sağlığının korunması, dişin uzun süreli sağlığı için çok önemlidir. Vital pulpa tedavisi travma, çürük veya diş prosedürlerinden etkilenen dişlerde pulpa dokusunun canlılığını korumayı ve desteklemeyi amaçlamaktadır. Birincil amaç, diş canlılığını korumak için ilave dentin oluşumunu teşvik etmektir. Vital pulpa tedavisindeki başarı; doğru teşhise, hasta seçimine ve pulpanın maruz kaldığı yer, pulpa olgunluğu ve mevcut diş tedavisinin kalitesi gibi çeşitli faktörlerin değerlendirilmesine bağlıdır. Vital pulpa tedavisinde, açıkta kalan dentin üzerine pulpa kaplama materyali adı verilen koruyucu bir biyoajan uygulanır. Başarılı doku yanıtlarını teşvik etmek ve hasta sonuçlarını iyileştirmek için çok sayıda diş biyomateryali geliştirilmiştir. Dentin-pulpa kompleksi iyileşme mekanizmalarına ilişkin anlayışımızdaki gelişmelerle birlikte, konservatif ve restoratif diş prosedürleri sırasında pulpa canlılığını destekleyen yeni biyomateryaller ortaya çıkmıştır. Ancak mevcut seçeneklerin çeşitliliği nedeniyle her klinik senaryo için en uygun biyomateryali seçmek zor olabilmektedir. Bu nedenle, pulpa kaplamanın diş sağlığının korunmasındaki önemli rolü göz önüne alındığında, yeni pulpa kaplama biyomateryallerinin biyoaktivitesinin geliştirilmesi ve değerlendirilmesi çok önemlidir. Bu derlemede, yeni pulpa kaplama biyomateryallerinin hem sitoprotektif hem de sitotoksik özelliklerini değerlendirmek için sıklıkla kullanılan tipik in vitro hücre kültürü ve moleküler biyoloji tekniklerini derlenmiştir. Bu teknikler, biyomateryal etkinliğinin ve güvenliğinin kapsamlı bir şekilde değerlendirilmesine katkıda bulunarak hayati pulpal tedavi uygulamalarının ve hasta bakımının geliştirilmesine yardımcı olur.

**Anahtar Kelimeler:** Pulpa kaplama biyomateryalleri, Biyoaktivite, Biyouyumluluk, Hücre Kültürü

## Introduction

Maintaining the health of the dental pulp is crucial for the lasting health of a tooth. Vital pulpal therapy (VPT) aims to safeguard and support the vitality of the pulp tissue in teeth that have been affected by traumatic exposure, caries, or dental procedures (aka mechanical injury).[1] The goal is to encourage the development of reperative dentin to keep the tooth as functional as possible. Preserving the dental pulp's vitality is essential due to factors such as the tooth's health, nourishment to the pulp, nerve supply, and immune system response mechanism. If the pulp exposure is not treated as it should be, it can lead to an infection in the pulp, necessitating more extensive treatment options like traditional root canal therapy or even tooth extraction.[1-3]

Accurate diagnosis and patient selection, identifying the location of pulp exposure, assessing the maturity of tooth roots, and ensuring the quality of the existing dental work are key factors for successful outcomes in VPT. In VPT, a protective bioagent, known as a pulp capping material, is applied over the remaining thin layer of dentin. VPT procedures vary in invasiveness, ranging from less intrusive methods like indirect and direct pulp capping to more extensive approaches such as partial or full pulpotomy.[1-3] Indirect pulp capping is a frequently employed technique in deep cavities containing a thin layer of healthy dentin above the pulp, with the goal of averting pulp exposure and further injury. This procedure can be performed using either a one-step or two-step method. In the one-step method, the majority of the carious dentin is removed, and a biomaterial is applied, ensuring it does not come into contact with the pulp, and the final restoration is completed in the same session. Conversely, the two-step approach involves gradual removal of caries, leaving firm, discoloured, deep carious dentin to avoid pulp exposure. A calcium hydroxide liner is then applied followed by a temporary restoration. Following several months of clinical observation, if no signs of pain or pathology are detected, the provisional restoration and any remaining caries needs to be removed, and then the final restoration can be applied.[1] Direct pulp capping (DPC) is seen as an effective and minimally invasive treatment choice. Following the removal of caries, a dental biomaterials is applied directly onto the exposed pulp to encourage the formation of mineralised tissue, commonly employed to safeguard the dental pulp's health.[1]

Over time, numerous dental biomaterials have been developed aiming to elicit the safest response from tissues and enhance patient outcomes. As our understanding of the healing mechanisms within the dentin-pulp complex has expanded, new biomaterials have emerged to support pulp vitality during conservative and restorative dental

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procedures. Given the array of biomaterial options for dental pulp capping, selecting the most suitable one for each clinical situation can pose a challenge. The perfect pulp capping biomaterial would possess various essential characteristics: simplicity in handling during procedures, adherence to dental surfaces, antibacterial and cytoprotective qualities, efficient sealing, durability against tissue fluid dissolution, biocompatibility, and bioactivity, promotion of mineralised tissue regeneration, and prevention of tooth discoloration.[1,2,4] Yet, there isn't a single dental pulp capping biomaterial that possesses all these desired traits at present. Nonetheless, recent years have witnessed notable progress in the development of these materials.

Assessing the bioactivity of novel pulp capping biomaterials is paramount due to the pivotal role pulp capping plays in maintaining dental health. When dentin is exposed due to caries or trauma, pulp capping serves to protect the pulp from further damage and promote its healing and regeneration. Thus, it is crucial to ensure that the materials used for pulp capping not only effectively seal the exposure but also possess properties that stimulate dentin regeneration and inhibit bacterial infiltration.[4,5] Furthermore, these materials should be biocompatible, meaning they do not elicit adverse reactions or inflammation within the dental pulp tissue. Inadequate bioactivity or biocompatibility of pulp capping materials could lead to complications such as pulp necrosis, infection, or even tooth loss.[5,6] Therefore, thorough evaluation of the biological response to these materials is essential to guarantee their safety and efficacy. In this context, employing in vitro cell culture-based procedures becomes increasingly prevalent. These methods allow researchers to simulate the interaction between cells and the novel biomaterials under controlled culture conditions. By examining parameters such as cell viability, proliferation, differentiation and death researchers can assess the biological response elicited by these materials and determine their potential for promoting pulp healing and tissue regeneration. Overall, comprehensive evaluation of the bioactivity of novel pulp-capping biomaterials through in vitro cell culture studies is essential for advancing dental care and ensuring the successful treatment of dental pulp exposures while minimising risks to patients' oral health. This review provides an overview of common in vitro cell culture and molecular biology techniques used to assess the cytoprotective and cytotoxic effects of pulp-capping biomaterials, including cell culture methods, viability assays, migration assays, cell death assays, and assays for biomineralisation and differentiation.

## Mammalian Cell Culture

### *Basics of mammalian cell culture techniques*

Cell culture is primarily associated with developing model systems for studying fundamental cell biology, replicating disease processes, or examining the bioactivity or cytotoxicity of novel compounds.[7-12] Culture systems are utilised to sustain cells in a manner that replicates the physiological environment found in living organisms, thus validating the clinical applicability of research findings. In mammalian cell cultures, efforts are made to replicate the conditions of extracellular fluids, encompassing adjustments in temperature to approximately 37°C, oxygen levels to 18.6% - 20.9%, carbon dioxide levels to 5%, and pH levels to 7.4 units. Ensuring the maintenance of pertinent physiological conditions within cell cultures is critical to ensure the consistency of reported findings and the applicability of experimental data to clinical scenarios.[7,13]

Maintaining aseptic technique is imperative when handling cell cultures to safeguard both the cultured cells and laboratory personel from potential infections. Aseptic cell culture practices involve a series of techniques and procedures aimed at maintaining a sterile environment during the handling and manipulation of cells in laboratory settings. The primary goal is to prevent contamination by unwanted microorganisms, such as bacteria, fungi, and viruses, which could compromise experimental results and potentially harm researchers.[14] All equipment, materials, and surfaces coming into contact with the cell culture must be sterilised before being employed. This typically involves techniques such as autoclaving (steam sterilisation), filtration, or treatment with certain chemical disinfectants. Researchers must wear appropriate personal protective equipment (PPE), including laboratory coats, gloves, and sometimes masks or goggles, to minimise the risk of introducing contaminants from the environment or their own skin. The cell culture work space, including the biosafety cabinet or laminar flow hood, incubator, cell culture benches, microscopy and centrifuges should be regularly cleaned and disinfected to maintain a sterile work environment. Only essential items such as culture media and other solutions, pipettors, pipet tips and microcentrifuge tubes should be present in the workspace during cell culture procedures. Careful handling of cell cultures and culture vessels is also crucial to minimise the potential contamination risks. Techniques such as flame sterilisation of tools, using sterile pipettes and media, and minimising exposure of culture vessels to the environment help reduce the risk of contamination. Following standardised protocols and procedures for basic and advanced cell culture, including thawing and frozen cells, passaging (subculturing) cells, and changing media, helps maintain consistency/reproducibility and minimise the risk of errors or contamination. Cultures should be routinely monitored for signs of contamination, such as changes in colour, turbidity, or growth patterns. Any contaminated cultures should be promptly discarded, and the affected area thoroughly cleaned and disinfected. Taken together, aseptic cell culture practices are essential for maintaining the integrity of cell cultures and ensuring reliable and reproducible experimental results in biological and biomedical research.[7,13-16]

### *Cell types used in dental investigations*

The primary culture refers to the initial in vitro cultivation of cells and tissues directly harvested from animals and humans. Often, these cultures display fundamental traits akin to their in vivo counterparts, making them valuable for basic research and various in vitro applications. While cells in certain primary cultures have the capability to proliferate and undergo subculturing in early passages, they typically exhibit a finite life span and tend to alter their differentiated traits over time in culture. Furthermore, primary cultures frequently comprise heterogeneous cell populations, posing challenges in standardisation and reproducibility due to uncontrollable variations between preparations.[14,17,18] Primary cell culture holds significance in biomedical research, as adjustments in culturing conditions can modulate the biological characteristics and functionality of cells, potentially rendering them more conducive for experimentation. Various approaches are employed, including the supplementation of culture media with diverse regulatory factors like cytokines, pharmaceuticals, and biological cell derivatives such as extracellular vesicles.[17,18]

The secondary cell culture consists of cells capable of prolonged multiplication in laboratory settings, thus enabling their maintenance through successive subculturing. These cell lines are categorised into finite, continuous, and stem cell lines based on their growth characteristics and potential for long-term culture. Finite cell lines refer to cell cultures capable of being subcultured multiple times; however, they eventually reach a state of senescence where cell replication halts, though the cells remain viable and may retain certain functional activities.[13,14] Some cell lines exhibit an apparent capacity for indefinite subculturing and are termed continuous cell lines. Continuous cell lines are commonly derived from either tumours or normal embryonic tissues. Last but not least, stem cell lines possess the defining properties of stem cells and have the ability to generate various specialised cell types. Yet, maintaining their stem cell attributes and differentiation potential necessitates meticulous attention in handling, maintenance, and preservation.[14,19]

The primary focus of dental research within the distinct connective tissue of dental pulp revolves around three main cell populations: odontoblasts, serving as the initial defence mechanism against injury; dental pulp stem cells (DPSCs), renowned for their robust self-renewal capacity and capability to differentiate into various mesodermal lineages; and dental pulp fibroblasts (DPFs), constituting the predominant cellular component within the dental pulp.[20,21] An escalating number of studies in dental research are utilising human DPSCs for evaluating the bioactivity of pulp capping biomaterials.[22-30] For instance, in a report published by López-García and his colleagues in 2019, a thorough comparison of the biological characteristics of ACTIVA Kids BioACTIVE Restorative (Pulpdent, USA) (Activa), Ionolux (Voco, Germany), and Riva Light Cure UV (SDI, Australia) was conducted utilising DPSCs [22]. Likewise, human DPFs have been utilised in numerous recent research studies to evaluate the bioactivity of emerging pulp-capping biomaterials in comparison to conventional options.[26,31-33] For example, Dou et al. (2020) employed DPFs to

investigate and compare the cytoprotective and cytotoxic effects of calcium hydroxide (Ca(OH)2), mineral trioxide aggregate (MTA), iRoot BP, platelet-rich fibrin (PRF) and concentrated growth factors (CGF).[33] Apart from primary DPSCs and DPFs, many other investigations have been using mammalian secondary cell lines such as L929 (NCTC clone 929, ATCC) [34,35] and NIH 3T3 [36] mouse fibroblast cells. Kato et al. (2023) assessed the biocompatibility of fast-setting calcium silicate-based pulp capping cements, namely Biodentine™, TotalFill® BC RRM™ Fast Putty, and Theracal LC®, using L929 fibroblast cells.[34] In summary, both primary DPSCs and DPFs, as well as secondary cell lines like L929 and NIH-3T3 fibroblast cells, present significant potential for exploring the biological properties of innovative pulp capping biomaterials.

*Production of Experimental Pulp Capping Material Disks*

To prepare pulp capping materials for bioactivity and biocompatibility assessments in accordance with ISO 10993-12:2021, it is crucial to standardise the size and shape of the test samples to ensure consistent and reliable results. ISO 10993-12:2021 is a part of the ISO 10993 series of standards, which provide guidance on the biological evaluation of medical devices. Specifically, ISO 10993-12:2021 outlines the methods for the preparation and handling of samples and reference materials for testing. [37] This standard ensures that biological evaluation tests are conducted on materials in a consistent and reproducible manner, allowing for reliable and comparable results across different studies and laboratories. When determining the sample sizes of the materials, it should be aimed for a surface-to-volume ratio of 3 $cm^2$/mL (ISO 10993-12:2021), and during the release phase, the culture medium should be able to completely surround the samples. All materials and equipment need to be sterilised to avoid contamination. The selected pulp capping material, such as calcium hydroxide, mineral trioxide aggregate (MTA), should be prepared and mixed thoroughly to ensure homogeneity. Sterile moulds could be employed to form the material into discs with the specified dimensions. The discs then are allowed to set or cure completely under conditions appropriate for the material type, ensuring that no residual monomers or other reactants remain, which could affect the test outcomes. Once the discs are prepared, they should be incubated in simulated cell culture media to evaluate ion release and hydroxyapatite formation, which are indicative of bioactivity. For biocompatibility assessments, in vitro cell culture tests can be conducted using relevant cell lines, such as human dental pulp cells. These tests should include direct and indirect contact assays, alongside viability assays (e.g., MTT, live/dead staining) to evaluate cytotoxicity, cell proliferation, and differentiation. The results are then statistically analysed to determine the material's suitability for clinical use, ensuring that all assessments adhere to the guidelines and specifications of ISO 10993-12:2021. [22,24,25,27,29,30]

**Cell Viability - MTT Assay**

Cell-based assays are commonly used to screen various compounds to determine whether the substances being evaluated affect cell proliferation or exhibit direct cytotoxic effects leading to cell death. Irrespective of the specific cell-based assay employed, it is essential to ascertain the remaining count of viable cells upon concluding the experiment.[38] There are various assay methods to determine the quantity of viable eukaryotic cells, among which tetrazolium reduction assays stand out. These assays assess enzymatic activity as a reliable indicator of cell viability. Tetrazolium salt solutions typically exhibit either a colourless appearance or faint coloration, but they undergo a significant transformation to form a strongly coloured solution upon the formation of the formazan product.[39] This assay involves incubating a reagent with a population of viable cells to convert a substrate into a coloured product, which can then be detected using a microplate reader (spectrophotometer, ELISA reader). In conventional cell culture environments, when viable cells are incubated with a substrate, the signal produced is directly proportional to the quantity of viable cells. As cells perish, they quickly lose their capacity to convert the substrate into a product. Various tetrazolium compounds have been employed for the detection of viable cells. Among the most frequently utilised compounds are MTT, MTS, XTT, and WST-1.[38-41] In this section, we provide a brief overview of MTT cell viability assays, focusing on their specific applications in dental research.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is the foremost and widely employed tetrazolium salt in molecular biology and biomedical research for assessing cell viability, proliferation, and cytotoxicity. In 1983, Mosmann first introduced MTT assay to assess proliferation and cytotoxicity in high-throughput screening methods conducted in multiwell culture plates. The assay relies on the conversion of MTT by viable cells into formazan crystals, indicating mitochondrial activity.[42] In other words, it is based on the principle that metabolically active cells possess functional mitochondria, which can reduce the yellow water-soluble MTT reagent to an insoluble purple formazan product. The assay involves incubating cells with MTT solution for a specific period (1 to 4 hours), allowing viable cells to convert MTT into formazan crystals. After the incubation period, the formazan crystals are solubilized using a solvent such as DMSO (dimethyl sulfoxide), and the resulting coloured solution is measured spectrophotometrically by an ELISA microplate reader. The intensity of the colour is directly proportional to the number of metabolically active cells.[39,43,44]

The MTT assay offers several advantages, including simplicity, speed, and cost-effectiveness. It requires minimal equipment and expertise, making it accessible to research in various fields, including dental investigations, such as in evaluating the biological profile of novel compounds. Nevertheless, it is important to note that the MTT assay has some limitations. It measures metabolic activity rather than cell viability per se, so factors influencing mitochondrial function can affect the results. Furthermore, the assay endpoint is influenced by various parameters such as cell density, incubation time, and the presence of interfering substances.[43-45] Despite these limitations, the MTT assay remains a widely used tool in cell biology, molecular biology and biomedical research for its versatility and reliability in assessing cell health and function.

To ensure reproducibility and reliability in MTT analysis, it is essential to strictly adhere to the assay protocol provided by the manufacturer of the reagents used. Initially, exponentially growing cells are counted and an equal number is seeded into a suitable cell culture vessel, like a 96-well microplate. These cells are allowed to adhere and proliferate to the desired confluency through at least overnight incubation. Following treatment with experimental compounds, if necessary, MTT solution is introduced to the cells and incubated for a specified period, typically a few hours, to facilitate the conversion of MTT into formazan crystals by metabolically active cells. Subsequently, the MTT solution is aspirated, and a solvent such as DMSO is added to dissolve the formazan crystals. The absorbance of the resulting solution is then measured spectrophotometrically at an appropriate wavelength, typically around 570 nm, using an ELISA microplate reader. Ultimately, the relative cell viability or metabolic activity is determined by comparing the absorbance readings of treated samples with appropriate controls. This meticulous adherence to the protocol ensures consistency and accuracy in MTT analysis outcomes.[43-45]

In their 2020 study, Kim and colleagues conducted a comparative analysis of the biological activity of different calcium silicate-based pulp capping biomaterials with conventional calcium hydroxide, utilising human DPSCs.[46] They conducted an MTT assay in order to assess the effect of ProRoot MTA (Dentsply Tulsa Dental Specialties, USA), Biodentine (Septodont, France), TheraCal LC (Bisco, USA,) and Dycal (Dentsply Caulk, USA) on cell viability. The material disks were inserted into inserts with a pore size of 0.4 µm and were subsequently cultured with attached hDPSCs for a duration of up to 5 days. After this incubation period, the researchers performed the MTT assay to evaluate cellular viability, revealing that, among other substances, Dycal exhibited the lowest cell viability even after a 1-day incubation [46]. In addition to MTT, other colorimetric assays such as XTT [47], CCK-8 [27], WST-1 [23] and MTS [28] are also extensively employed in dental research investigations.

**Cell Migration - Wound-healing Assay**

A wound-healing assay (aka 'scratch assay') is a commonly used experimental technique in cell biology, molecular biology and biomedical research. It is designed to study the process of cell migration and wound closure in vitro.[48] The assay involves creating a "wound" (gap, scratch) in a monolayer of cultured cells, typically using a sterile pipette tip or a specialised tool to scratch the cell layer. This creates a well-defined area devoid of cells, mimicking a wound in vivo. After the scratch is made, the progress of wound closure is monitored and recorded over time using time-lapse microscopy or by capturing images at regular intervals.[49] The experiment usually extends for up to 24 hours

because beyond this timeframe, not only migration but also proliferation play significant roles in facilitating cell-mediated wound closure. As cells migrate into the wound area to close the gap, the width of the scratch decreases, allowing researchers to quantify the rate of cell migration and wound closure.[50]

Wound-healing assays are valuable tools for studying various cellular processes involved in wound repair, including cell migration, proliferation, adhesion, and cytoskeletal dynamics. They can be used to investigate the effects of different factors such as growth factors, cytokines, drugs, or genetic manipulations on wound healing mechanisms. Additionally, these assays offer valuable insights into assessing the bioactivity of newly developed compounds intended for dental treatments.[22,24-27,47] Overall, wound-healing assays are versatile and widely used techniques that offer valuable information about cellular behaviour and tissue repair processes, making them essential tools in biomedical research.[48-51] To assess the influence of ACTIVA Kids BioACTIVE Restorative, Ionolux, and Riva Light Cure on cell migration, López-García et al. (2019) undertook a comprehensive wound-healing assay employing human DPSCs.[22] By employing this experimental setup, they aimed to evaluate the potential effects of these dental materials on the migratory behaviour of DPSCs, providing valuable insights into their suitability for dental applications.

In brief, a predetermined number of cells, sufficient to achieve full confluency the following day, are seeded onto multiwell plates, typically 6-well plates, and cultured until they form a uniform cell monolayer. Subsequently, each well is carefully scratched using a sterile pipette tip, followed by several washes with PBS to remove any cellular debris. The healing process is then allowed to progress in the presence of various material eluates or in the absence of eluates (e.g. control group). The extent of wound closure is assessed at multiple time intervals, such as 6, 12, and 24 hours. The width of the scratch is quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) to determine the percentage of wound area at different time points.[12,48]

**Apoptosis and Necrosis - Annexin V Assay**

The Annexin V fluorescence-activated cell sorting (FACS) assay is a molecular biology technique used to detect and quantify apoptotic cells within a cell population. It involves staining cells with fluorescently labelled Annexin V, a protein that binds specifically to phosphatidylserine, a membrane phospholipid that becomes exposed on the outer leaflet of the plasma membrane during apoptosis.[52] Additionally, a DNA-binding dye such as propidium iodide (PI) may be used to differentiate between apoptotic and necrotic cells.

In the assay, cells are typically treated with various experimental conditions or compounds known to induce apoptosis. Following treatment, the cells are harvested, washed, and incubated with Annexin V and PI. Fluorescence-activated cell sorting (FACS) analysis is then performed to quantify the proportion of cells that are Annexin V-positive (indicating early apoptosis), PI-positive (indicating late apoptosis or necrosis), or double-positive for both markers. The Annexin V FACS assay provides valuable information about the apoptotic status of cells in response to different stimuli, allowing researchers to study apoptotic pathways, screen for potential therapeutic agents, or assess drug efficacy.[52-54]

The Annexin V assay finds applications in dental investigations as well, particularly in studies related to dental pulp and periodontal tissues. Researchers utilise this assay to investigate the apoptotic response of dental pulp cells, periodontal ligament cells, or gingival fibroblasts to various stimuli such as bacterial infection, inflammatory cytokines, or dental compounds including pulp capping biomaterials.[26,30,33] By assessing apoptotic cell death in these tissues, researchers can gain insights into the mechanisms underlying dental diseases such as pulpitis, periodontitis, or periapical lesions. Additionally, the Annexin V FACS assay is employed to evaluate the cytotoxic effects of dental materials, including restorative materials, endodontic sealers, or orthodontic adhesives, providing crucial information for assessing their biocompatibility and safety in clinical applications.

**Biomineralisation - Alizarin Red Staining Assay**

The Alizarin Red staining assay is a widely cell biology technique used to detect and quantify calcium deposits, particularly in cell culture models of osteogenesis and mineralisation.[55] It involves the use of Alizarin Red dye, which binds to calcium ions, forming a complex that can be visualised and quantified using spectrophotometry or microscopy. In the assay, cells are cultured under conditions that promote osteogenic differentiation and mineralisation, such as the addition of specific growth factors, mineralisation-inducing media supplements or compounds. After a defined period of culture, the cells are fixed and stained with Alizarin Red dye. Calcium deposits within the cell monolayer will bind to the dye, resulting in a characteristic red-orange staining. The stained cells can then be visualised under a microscope, and the intensity of staining can be quantified using image analysis software or by extracting the dye and measuring its absorbance spectrophotometrically.[55,56] The amount of Alizarin Red staining correlates with the extent of calcium deposition, providing a qualitative and quantitative assessment of osteogenic differentiation and mineralisation capacity in vitro. The Alizarin Red staining assay is commonly used in research related to bone biology, tissue engineering, regenerative medicine and dental investigations to evaluate the osteogenic potential of cell populations or the efficacy of therapeutic interventions aimed at promoting bone formation.

Biomineralisation is a significant functional characteristic of pulp capping biomaterials. When dental caries or traumatic injuries damage the dentin-pulp complex, clinical interventions should aim to facilitate the regeneration of pulp tissue that is functionally competent. This regenerated tissue should be capable of promptly forming mineralised tissue to restore lost structure and effectively seal the clean pulp environment from potential contamination by the external oral environment [57]. In recent times, several studies have been conducted to assess whether newly developed bioactive pulp capping biomaterials have the ability to enhance cellular biomineralisation.[27,28,58,59] Jun and colleagues (2017) conducted a comparative analysis of the cytotoxicity and biomineralisation potential of a newly developed bioactive glass-incorporated light-curable pulp capping material. They compared it with a chemically curable product incorporating calcium hydroxide, serving as the gold standard control for calcium release. Additionally, they assessed a light-curable pulp capping material incorporating MTA-like components as the counterpart to the light-curable material under investigation.[28] Taken together, there are many studies employing the Alizarin Red staining assay in order to evaluate the cellular biomineralisation potential of newly developed bioactive pulp capping biomaterials, aiming to facilitate the regeneration of functionally competent pulp tissue capable of promptly forming mineralised tissue to restore lost structure and seal the clean pulp environment.

**Gene Expression - quantitative PCR (qPCR) Analysis**

Polymerase Chain Reaction (PCR) is a molecular biology technique used to amplify a specific segment of DNA. It involves multiple cycles of heating and cooling to denature the DNA, anneal primers to the target sequence, and extend new DNA strands using a DNA polymerase enzyme. PCR enables the rapid and precise amplification of DNA, making it invaluable in various applications such as genetic testing, cloning, and sequencing.[60] Quantitative real-time PCR (qPCR), also known as real-time PCR or quantitative PCR, is an advanced molecular biology technique used to quantify the amount of a specific DNA or RNA sequence in a sample. Unlike conventional PCR, which only provides qualitative information about the presence or absence of a target sequence, qPCR allows for the precise measurement of target nucleic acid molecules in real-time during the amplification process.[60,61] In qPCR, fluorescent dyes or probes are used to monitor the accumulation of PCR products as they are produced in each cycle of amplification process. The fluorescence signal increases proportionally to the amount of PCR product, providing a quantitative measurement of the initial amount of target nucleic acid in the sample. qPCR assays encompass two primary types: SYBR Green and probe-based assays. In SYBR Green assays, the double-stranded DNA-binding dye SYBR Green is employed to detect PCR products. The dye fluorescence intensifies upon binding to newly synthesised double-stranded DNA during PCR amplification. A qPCR instrument monitors this fluorescence in real-time, and the threshold cycle (Ct) value, indicating the cycle number at which the fluorescence signal surpasses a predetermined threshold, is employed to quantify the initial amount of target nucleic acid in the sample.[61,62] In probe-based qPCR assays, fluorescently labelled probes are designed to specifically anneal to the target sequence during PCR amplification. These probes typically

consist of a fluorophore at one end and a quencher at the other end. During PCR amplification, the probe is cleaved by the DNA polymerase enzyme, resulting in the release of the fluorophore from the quencher and an increase in fluorescence signal. The fluorescence signal is measured in real-time by a qPCR instrument, and the Ct value is used to quantify the initial amount of target nucleic acid in the sample.[61-64] qPCR is widely used in various research and clinical applications, including gene expression analysis, pathogen detection, genetic testing, and molecular diagnostics. It offers high sensitivity, specificity, and dynamic range, making it a powerful tool for accurately quantifying nucleic acids in diverse biological samples. It is also commonly used to analyse gene expression profiles of key signalling pathways associated with odontogenic differentiation and mineralisation in dental pulp cells treated with different biomaterials[27,29,47,58], providing insights into the molecular mechanisms underlying tissue regeneration and repair. In their study, Widbiller and colleagues (2016) examined the biocompatibility and capacity to stimulate differentiation and mineralisation of Biodentine™ by evaluating the expression levels of biomineralisation-associated genes, specifically *COL1A1* (collagen type I alpha 1), *ALP* (alkaline phosphatase), *DSPP* (dentin sialophosphoprotein), and *RUNX2* (RUNX family transcription factor 2), in human DPSCs.[29] In a separate investigation, researchers explored the impact of iRoot Fast Set root repair material (iRoot FS) on the biomineralisation and differentiation of human DPSCs by analysing the mRNA expression of differentiation gene markers, namely *COL1A1* and *OCN* (osteocalcin).[27] Pedano et al. (2018) investigated the potential of freshly-mixed and setting calcium-silicate cements to stimulate human DPFs by examining specific markers of odontoblastic differentiation, such as *OCN*, *DSPP* (dentin sialophosphoprotein), and *DMP1* (dentin matrix protein 1), using qPCR analysis.[47]

**Conclusion**

The investigation of novel pulp capping biomaterials is pivotal in dentistry for several nuanced reasons. Firstly, these biomaterials possess regenerative potential, stimulating the natural healing process of the dental pulp and promoting the formation of new dentin and supporting tissue regeneration. Secondly, rigorous evaluation ensures the biological compatibility of these biomaterials with the surrounding dental tissues, minimising adverse reactions and inflammation while promoting tissue healing and reducing the risk of post-treatment complications. Thirdly, effective pulp capping biomaterials provide a durable seal that protects the pulp from bacterial invasion and further damage, maintaining the integrity of the pulp environment and preventing the spread of infection. Moreover, research efforts focus on developing biomaterials with enhanced longevity and sustainability, contributing to the preservation of tooth structure and functionality and reducing the need for frequent interventions. Additionally, the translation of scientific discoveries into real-world applications bridges the gap between laboratory research and clinical practice, facilitating personalised treatment options tailored to the specific needs of patients. In addition to conventional histological and functional assays, the molecular biology techniques outlined in this review enhance our comprehension of the biological reactions to emerging pulp-capping biomaterials, thus directing the advancement of more efficient regenerative treatments in dentistry.

In summary, the exploration of novel pulp capping biomaterials encompasses a multifaceted approach addressing biological, clinical, and patient-centred considerations, driving innovation in restorative dentistry and improving treatment outcomes.

**Değerlendirme / Peer-Review**

İki Dış Hakem / Çift Taraflı Körleme

**Etik Beyan / Ethical statement**

Bu makale, sempozyum ya da kongrede sunulan bir tebliğin içeriği geliştirilerek ve kısmen değiştirilerek üretilmemiştir.

Bu çalışma, yüksek lisans ya da doktora tezi esas alınarak hazırlanmamıştır.

Bu çalışmanın hazırlanma sürecinde bilimsel ve etik ilkelere uyulduğu ve yararlanılan tüm çalışmaların kaynakçada belirtildiği beyan olunur.

This article is not the version of a presentation.

This article has not been prepared on the basis of a master's/ doctoral thesis.

It is declared that during the preparation process of this study, scientific and ethical principles were followed and all the studies benefited are stated in the bibliography.

**Benzerlik Taraması / Similarity scan**

Yapıldı – ithenticate

**Etik Bildirim / Ethical statement**

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Makalenin Yazımı | Writing up: EBG (%30), RG (%70)
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