DENTAL MATERIALS 30 (2014) 769–784

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.intl.elsevierhealth.com/journals/dema

Methods to evaluate and strategies to improve the biocompatibility of dental materials and operative techniques

Carlos A. de Souza Costa[a,*], *Josimeri Hebling*[b], *Débora L.S. Scheffel*[b], *Diana G.S. Soares*[c], *Fernanda G. Basso*[a], *Ana Paula D. Ribeiro*[d]

[a] *Department of Physiology and Pathology, Araraquara School of Dentistry, UNESP – Univ. Estadual Paulista, Araraquara, São Paulo, Brazil*

[b] *Department of Orthodontics and Pediatric Dentistry, Araraquara School of Dentistry, UNESP – Univ. Estadual Paulista, Araraquara, São Paulo, Brazil*

[c] *Department of Dental Materials and Prosthodontics, Araraquara School of Dentistry, UNESP – Univ. Estadual Paulista, Araraquara, São Paulo, Brazil*

[d] *Department of Dentistry, Universidade Federal de Brasília – UNB, Brasília, Brazil*

ARTICLE INFO

Article history:
Received 26 August 2013
Received in revised form
5 February 2014
Accepted 25 April 2014

Keywords:
Cytotoxicity
Biocompatibility
Dental materials
Dentin
Dental pulp
Biological response

ABSTRACT

Objective. The general aim of this article is to describe the state-of-the-art of biocompatibility testing for dental materials, and present new strategies for improving operative dentistry techniques and the biocompatibility of dental materials as they relate to their interaction with the dentin–pulp complex.

Methods. The literature was reviewed focusing on articles related to biocompatibilty testing, the dentin–pulp complex and new strategies and materials for operative dentistry. For this purpose, the PubMed database as well as 118 articles published in English from 1939 to 2014 were searched. Data concerning types of biological tests and standardization of *in vitro* and *in vivo* protocols employed to evaluate the cytotoxicity and biocompatibility of dental materials were also searched from the US Food and Drug Administration (FDA), International Standards Organization (ISO) and American National Standards Institute (ANSI).

Results. While there is an ongoing search for feasible strategies in the molecular approach to direct the repair or regeneration of structures that form the oral tissues, it is necessary for professionals to master the clinical therapies available at present. In turn, these techniques must be applied based on knowledge of the morphological and physiological characteristics of the tissues involved, as well as the physical, mechanical and biologic properties of the biomaterials recommended for each specific situation. Thus, particularly within modern esthetic restorative dentistry, the use of minimally invasive operative techniques associated with the use of dental materials with excellent properties and scientifically proved by

* *Corresponding author at*: Faculdade de Odontologia de Araraquara, Rua Humaitá, 1680, Araraquara, São Paulo 14801-903, Brazil. Tel.: +55 16 3301 6477; fax: +55 16 3301 6488.
E-mail address: casouzac@foar.unesp.br (C.A. de Souza Costa).

http://dx.doi.org/10.1016/j.dental.2014.04.010

means of clinical and laboratory studies must be a routine for dentists. This professional and responsible attitude will certainly result in greater possibility of achieving clinical success, benefiting patients and dentists themselves.

Significance. This article provides a general and critical view of the relations that permeate the interaction between dental materials and the dentin–pulp complex, and establish real possibilities and strategies that favor biocompatibility of the present and new products used in Dentistry, which will certainly benefit clinicians and their patients.

1. Introduction

There has been much discussion about new proposals for pulp tissue regeneration therapy. For several decades researchers have related that the acquisition of more in-depth knowledge of molecular biology and the mechanisms involved in the process of repair and regeneration of the dentin–pulp complex may direct new treatment strategies for pulps submitted to various types of aggressions, such as contamination, trauma, cytotoxicity of dental material components, and thermal injuries. This has appeared to become more evident and possible by means of the recent advancements in knowledge of the functions and activities of pulpal stem cells and signaling molecules present in dental tissues [1,2]. However, applying this body of knowledge and the promising results obtained in laboratory researches to clinical situations has been a great challenge.

The incessant search for new knowledge that direct future therapies associated with a more biological approach that allows and/or stimulates pulp repair has, up to a certain point, led to neglect in evaluating the dentin–pulp complex response to the different clinical procedures being used at present. This becomes evident when new dental materials and operative techniques are recommended for certain therapies without these having been scientifically evaluated, so that they are effectively shown to be safe for all patients. Proof of this was the recommendation, about 15 years ago, to etch the exposed pulp tissue with acidic agents and proceed with direct pulp capping with bonding agents [3,4]. This type of therapy, in spite of having been scientifically demystified some years ago [5,6], has been the focus of some roundtable discussions even today, in which some consider this therapy a promising clinical procedure capable of stimulating the repair of exposed pulps [7]. In addition, one modality of an esthetic clinical procedure that presents limited evaluation with respect to possible damage to the dentin–pulp complex is dental bleaching. This type of therapy has been widely used all over the world, even knowing that around 70–80% of the patients submitted to dental bleaching have related some type of pain or discomfort during and/or post-treatment [8–10]. What would be the reason for this undesirable side effect caused by bleaching vital teeth? Why does post-bleaching pain regress and disappear within some days? Would it not be interesting to research this clinical procedure and understand the possible damage caused to the dentin–pulp complex by the different techniques and products used in dental bleaching?

Logically, researches must be conducted to establish a certain therapy as being safe for patients and for dentists themselves. From then on, it may be recommended for clinical application, with this maxim being true for any new dental material or new recommendation for treatment in the health area. This is the guideline of International Organizations, such as the US Food and Drug Administration (FDA) and International Organization of Standardization (ISO), which determine the performance of rational tests *in vitro*, in animals and usage/clinical tests, which must be adapted to present days [11]. Undoubtedly, the development and the constant search for knowledge within the topic of tissue repair are relevant as a whole, and certainly will, in the very near future, direct important modalities of therapy for the dentin–pulp complex. Therefore, the main objective of this article is to provide a general and critical view of the relations that permeate the interaction between dental materials and the dentin–pulp complex, and establish real possibilities and strategies that favor biocompatibility of the present and new products used in Dentistry, which will certainly benefit clinicians and their patients.

2. Biocompatibility tests for dental materials

In 1970, Autian [12] proposed a sequence of studies to determine the safety of the clinical use of new materials. The purpose of conducting *in vitro* researches, followed by investigations in animals, and finally, clinical studies is to evaluate the biocompatibility of new materials in an ethical and financially feasible manner, and at every level, eliminate materials that present greater cytotoxic potential. Nevertheless, results obtained *in vitro* may diverge from those observed in animals and humans. Therefore, the great challenge to laboratory tests at present, whether *in vitro* or in animals, is to seek greater approximation to clinical reality (for further details, we recommend reading the work of Wataha [11] and Bayne [13]).

2.1. *In vitro studies*

Analysis of the biocompatibility of materials *in vitro* occurs outside of live organisms, using cell cultures or constituents. These tests allow the incorporation of strategies to increase the proximity to clinical practice, such as for example, the use of physical barriers, such as enamel and/or dentin discs. The evolution of these methodologies allows for evaluation of cell function and viability, gene expression, the expression of proteins and inflammatory mediators, reactive oxygen species, type of cell death, cell morphology, among others [14–16].

Furthermore, *in vitro* studies enable greater reproducibility, speed, low cost, ease in determining control groups and avoid legal and ethical conflicts, such as submitting animals or humans to pain, suffering and possible risks in general [17].

The laboratory methodologies established some decades ago, and which continue be used nowadays, have been extensively criticized. For this reason, the *in vitro* research models have evolved significantly with the aim of being better able to mimic *in vivo* conditions [11].

The insertion of models that use a dentin barrier has been recognized as an important evolution of *in vitro* tests of dental materials biocompatibility, providing greater similarity of the experimental conditions to those observed *in vivo* [18,19]. Thus, the use of the artificial pulp chamber (APC) [14,15,20,21] is an effective experimental model for performing different laboratory protocols for the purpose of evaluating new materials and their application techniques (Fig. 1). For this *in vitro* model, dentin and/or enamel discs, which may be selected and standardized according to the object understudy, are inserted into APCs. In this protocol, the pulpal surface of the disk is kept in contact with the culture medium, and the occlusal surface remains exposed, so that the materials and/or technique can be applied and evaluated [15]. Particularly for *in vitro* tests in which dentin discs are used, an important advantage inherent to this methodology is that the samples, which may be obtained from human teeth or those of animals, may also be selected and standardized as regards thickness and permeability, factors that are directly related to the diffusion of toxic components related from the experimental materials under analysis [19,22,23].

The use of APCs also enables different stages of clinical procedures to be performed, such as acid etching and the application of adhesive systems before the application of restorative materials, among other techniques [14,21]. Moreover, it is also possible to perform tests with light polymerizable and chemically setting materials concomitantly with contact of the materials with the dentin substrate, and contact of the latter with the cell lineage to be evaluated [14]. Other models of APCs allow the application of intra-pulpal pressure, which may influence the conditions of application and solubility of the test materials, as well as the transdental diffusion of their components.

Another important parameter that should be taken into consideration for the development of *in vitro* studies, which must as far as possible, approximate and mimic the clinical conditions, is the selection of cell lineages that must be relevant to the analysis of the dental materials [24–26]. For the evaluation of dental materials and techniques for the application of new products indicated for use on different dental tissues, it is important to select cells that present an odontoblastic phenotype, because in the teeth of mammals this type of cell is organized in layers to line the coronal and root dentin internally (Fig. 2a and b). Within this context, the odontoblasts are the first pulp cells to come into contact with the components of dental materials capable of diffusing through the dentin and/or enamel/dentin. Therefore, cells of an odontoblastic lineage, such as the MDPC-23 cells, which were obtain from rat teeth by Hanks et al. [27], have been widely used for laboratory tests of new materials and techniques [14,15]. For these tests, undifferentiated pulp cells (OD-21) [28]; primary cultures of human pulp cells [29,30], or undifferentiated cells from human pulp [30] have also been used, which may more safely indicate the possible effects of the tested materials [31].

It should also be pointed out that a large portion of the *in vitro* cytotoxicity/biocompatibility studies have been developed with the use of monolayer cell culture models [15,16]. However, the 3D culture models, in which cells are cultivated in specific types of collagen matrix (scaffolds), appear to provide more favorable conditions for the morphological and phenotypical expression of the cells, and this experimental model may also be used for the direct and indirect evaluation of the biologic effects of new dental materials and techniques [24,32].

Another factor that contributes to the efficacy of the *in vitro* cytotoxicity/biocompatibility tests for dental materials is the selection of the tests to be performed [11], which may vary according to the type of product to be tested and the following levels of response one wishes to obtain: cytotoxicity, the induction of an inflammatory responses, biostimulation or cell differentiation capacity, among other important cellular functions for the homeostasis and repair of the dentin–pulp complex.

When considering cytotoxicity tests, the most widely used methods are those that determine cell viability, particularly by means of analyzing the mitochondrial activity of the cells exposed to the materials and/or their isolated components [14,16,31]. Other tests in turn, evaluate the occurrence of cell death [33] and whether this was due to necrosis or apoptosis [16]. These analyses may contribute to understanding the intensity of the toxic effects of dental materials, as those that induce apoptosis are considered less aggressive when compared with materials that induce cell death by necrosis [34]. Another parameter that may also be evaluated is the production of reactive oxygen species, which is directly related to the induction of cell lesion, and may be detected by means of fluorescent probes [35].

The application of cell metabolism tests may indicate stimulation or inhibition of cell differentiation, such as protein production and gene and protein expression of specific molecules, such as Collagen Type I and alkaline phosphatase, responsible for the formation and mineralization of the dentin matrix, respectively [36]. The protocol for analyzing the production of mineralized nodules may also be used to determine the induction or inhibition of the mineralization capacity of cells in culture after contact with different materials and/or their components [30]. On the other hand, laboratory methods that evaluate inflammatory cytokine expression by these cells in culture may indicate the possible *in vivo* induction of an inflammatory response in the pulp tissue [37].

In addition to quantitative tests, the effects of dental material on cell cultures may also be evaluated in a qualitative manner, such as in complementary analyses of cells by scanning electron microscopy [14,15]. By means of this method, it is possible to verify morphological changes caused by the materials and/or their isolated components, as well as to determine whether there was a reduction or increase in the population of cells adhered to a substrate after they

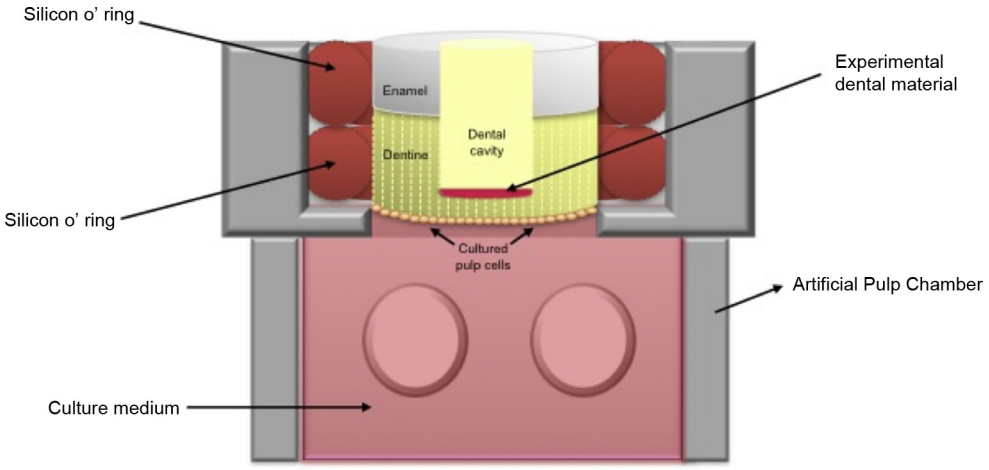

Fig. 1 – Schematic representation of an artificial pulp chamber.

had been exposed to the products being tested (Fig. 3a and b).

A third determinant factor for *in vitro* cytotoxicity/biocompatibility studies of dental materials is the selection of adequate concentrations and volumes of these products and their components, which must be close to those of clinical conditions [11].

In general, in spite of the limited clinical significance of scientific data obtained by *in vitro* researches, it is important to point out that the results of laboratory studies, if evaluated attentively and judiciously, may direct future *in vivo* investigations to be conducted in animals, and contribute significantly to the understanding of the data observed in clinical studies [11].

2.2. *In vivo studies (in animals)*

The use of animals for research is a controversial subject and has been the target of broad discussions, mainly of an ethical nature [38,39]. Nevertheless, these studies may provide more relevant scientific data than those observed *in vitro* and enable the evaluation of important parameters, such as the interaction of the material with blood, chronic inflammatory responses and bone regeneration [40]. Researches using

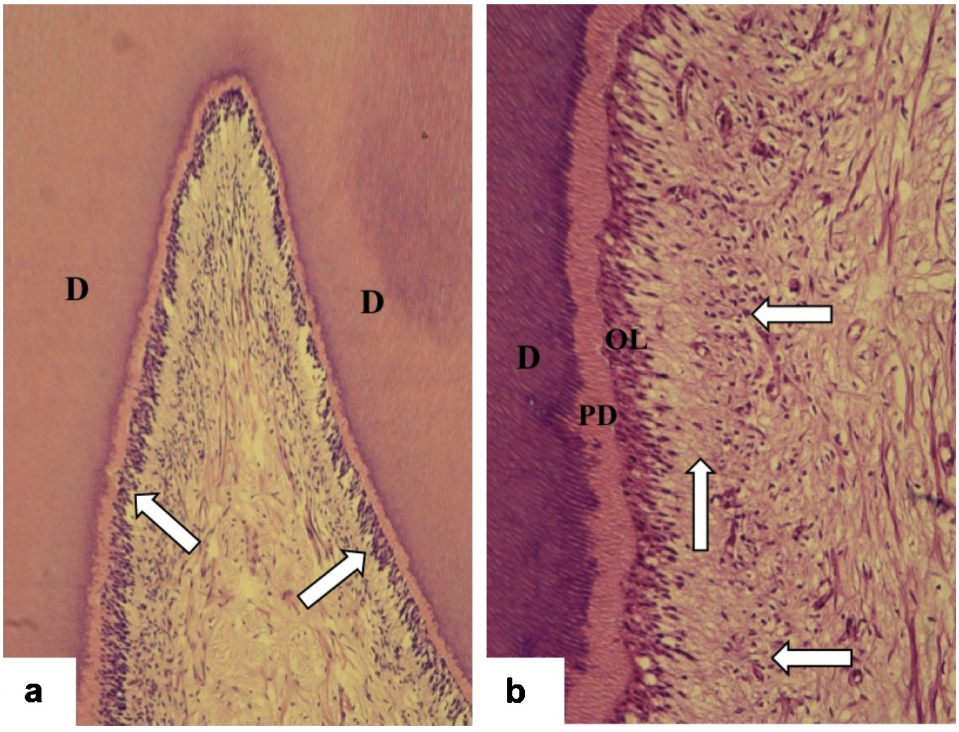

Fig. 2 – (a) MDPC-23 cells are attached to dentin substrate on which they were cultured; SEM × 500. (b) High magnification of Fig. 2a. Note the morphology of the pulp cells which exhibit a number of short and thin cytoplasmic processes; SEM × 1000.

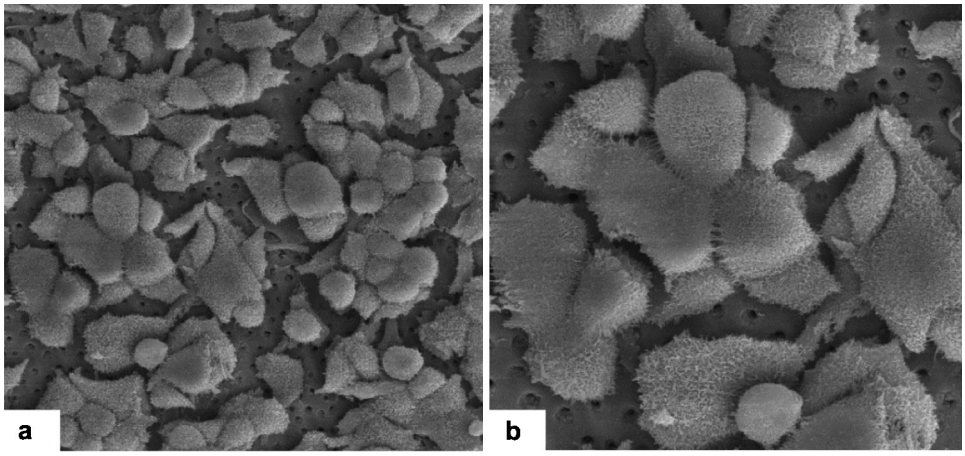

Fig. 3 – (a) Pulp horn of a human sound tooth. Note that a continuous odontoblast layer (arrows) is underlying the dentin (D); H/E, 64×. (b) Detail of the dentin–pulp complex. Note the tubular dentin (D), predentin (PD), odontoblast layer (OL), cell-free zone (vertical arrow) and the cell-rich zone (horizontal arrows). H/E, 125×.

laboratory animals present lower costs than clinical studies and may be satisfactorily controlled. Nevertheless, the results obtained in these tests may be influenced by the species, age and gender of the animal used in the studies [40] and cannot automatically be extrapolated to clinical situations in humans [11,41]. Moreover, interpretation of the responses observed is complex, since various events occur simultaneously, such as the trauma generated by the applications of the test materials in contact with the animal's tissues and possible local infections.

Studies in animals may, in a preliminary manner, determine the safety of using the material being tested, and predict its clinical success in a specific function [11]. In the first case, some specific parameters, such as dosage and administration pathway do not represent circumstances of clinical relevance; however, the main proposal is to expose the animal to extreme conditions that will allow the safety of the material being analyzed to be determined. Whereas, in the second case, the tests attempt to reproduce, in the best possible manner, the conditions found in clinical reality, such as size, dosage, method of application, presence of physical barriers, such as dentin and/or dentin/enamel, as well as the composition and manipulation of the materials [11].

Another difficulty found with regard to the use of animals for research is in determining adequate control groups that favor interpretation of the results, with the lowest possible number of biases. In addition, statistical analysis of the responses characterizes a great challenge, since there may be problems with respect to the correct definition of independent variables.

2.3. *Clinical studies*

Clinical studies are characterized by the application of the experimental materials in human volunteers and are considered the "gold standard" for the evaluation of properties and performance of dental materials [42]. These types of studies may present various experimental protocols that vary as regards cost and the difficulty of conducting them [43]. However, the large majority of clinical studies have sought to evaluate the mechanical properties of materials, so that biocompatibility would not be their main focus [44]. In general, clinical studies are more expensive, take longer, are more difficult to control and interpret the results, particularly when compared with researches developed in animals and *in vitro* tests. In addition, these studies using human beings face strong ethical barriers [45].

Among the different types of clinical studies, there are retrospective, cross-sectional and prospective studies. Longitudinal or prospective studies are more representative with regard to determination of the biological performance of a material, and there are strategies that can be used to increase their reliability, such as blinding, randomization, placebo groups and strategies to minimize biases [46]. After the treatment, the patients are followed-up over the course of time, which allows data collection. Nevertheless, in general, these types of studies are expensive, require a long period for their finalization and may be influenced by the operator's skill, which may be far above or below the clinical average [42,43].

2.4. *Correlation between biocompatibility tests*

Unfortunately, it is frequently not possible to obtain strong correlation between laboratory observations and the set of clinical properties in the short or long term [13]. This occurs for various reasons, among them the fact that laboratory tests are static, and on the other hand, analysis of clinical properties involves dynamic observations, such as change in the material over the course of time which, in spite of also occurring *in vitro*, does not necessarily reflect the same conditions as those observed *in vivo* [40].

In spite of the challenges found in correlating *in vitro* studies in animals and clinical studies, the regulatory agencies recognize the importance of laboratory studies in the evaluation of the biocompatibility of dental materials [47]. As previously mentioned, these tests must be as close as possible to the clinically relevant conditions, which range from the use of physical barriers through to the selection of the cell

type to be used and its condition of exposure to the products being tested. For studies in animals, the choice of species, site of application of the experimental material and time intervals of evaluation must also be taken into consideration [48,49].

It is known that the most efficient manner of evaluating the biocompatibility of a material is by means of the association of the results of *in vitro* tests with those obtained in animals and clinical tests [50]. Nevertheless, the manner in which this association of the scientific data should occur continues to be the area of many discussions and a challenge to be overcome, because the sequence of tests does not always present good correlation, particularly due to the limitations of laboratory tests in reproducing clinical conditions [51,52]. However, *in vitro* test are most useful for the initial determination of the cytotoxic potential of dental materials and for investigating specific mechanisms of the cell response to products and therapies.

In a recent literature review, Wataha [11] proposed a new strategy for the use of laboratory and clinical tests, in which *in vitro* studies would mainly be used for the evaluation and characterization of the constituents released by the materials and their dangers and risks, since the majority of the undesirable effects are caused by toxic substances related from these products. Tests in animals would have the main functions of complementing the *in vitro* tests and evaluate the risks and dangers of new products, determining their safe application. Thus, these tests may determine the main risks of the material (cytotoxicity, genotoxicity, carcinogenicity, *etc.*), so that from these initial data, safer clinical tests may be performed.

3. Dentin–pulp complex

Although dentin and pulp present distinct structural characteristics, these tissues arising from the same embryonic origin are intimately related and interdependent, so that both are recognized as a functional unit, denominated the dentin–pulp complex (DPC) [53,54]. Therefore, the development of strategies with the aim of increasing the biocompatibility of new dental materials and clinical procedures, maintaining the functional activity and vitality of the DPC, are directly related to knowledge of the biology and physiology of these tissues, and understanding of their inter-relations.

The DPC is a highly dynamic structure, capable of adapting to different stimuli that may trigger an inflammatory reaction, either associated with the synthesis and deposition of dentin matrix, or not [55,56]. The main link of communication between the dentin and pulp are the odontoblasts, which are responsible for the deposition and maintenance of dentin and are also involved in transmission of sensory stimuli in the DPC and cellular defense against pathogens [57]. These highly specialized cells, which present the main nucleus and organelles located in the cell body, are organized in a layer at the periphery of the pulp tissue, so that they internally line the coronal and radicular dentin. Cytoplasmic prolongations originated from the odontoblasts may be found within the dentinal tubules. Thus, a cavity preparation in dentin may lead to cutting off many odontoblast prolongations, which determines that this operative procedure must be judiciously and very carefully performed. The organization of odontoblasts at the periphery of the pulp and the presence of effective junctional complexes among these cells, make the odontoblastic layer act as a "semipermeable barrier" that allows only the exudation of interstitial fluid and low molecular weight proteins into the tubules, forming the dentinal fluid; thus, dentin physiology provides this tissue with two basic characteristics: permeability and humidity [53,54].

It is known that the diameter and quantity of tubules per area of dentin increase according to proximity to the pulp, which results in deep dentin presenting greater permeability, and consequently being more humid than superficial dentin [54]. These morphological differences of dentin have a direct relationship with the strategy to be defined for the maintenance of viability and homeostasis of the DPC, when different operative procedures are performed. Dentin permeability regulates the rate of diffusion of toxic products (bacterial or those released from dental materials) in the direction of the pulp tissue, which may determine the pattern and intensity of tissue response. It has also been demonstrated that the humidity present in dentin contributes to the resilience of this tissue, which is important for the distribution of stress resulting from masticatory forces [53]. Nevertheless, this characteristic of dentin may have adverse effects on the dental materials applied to this tubular and humid tissue [1]. Therefore, restorative procedures to be applied to exposed deep dentin tissue have become a challenge, particularly with regard to the advance of Adhesive Restorative Dentistry [41,58]. In order to go more deeply into knowledge of dentin tissue, we suggest reading the work of Tjärderhane et al. [59].

In addition to the primordial function of the odontoblasts in the synthesis and deposition of dentin matrix, it is known that these specialized pulp cells also act in the modulation of immune and inflammatory pulp responses, and are therefore considered the first line of defense of the DPC [57,60]. It has been demonstrated that the odontoblasts, which have receptors for the recognition of molecular patterns associated with pathogens, are capable of expressing inflammatory cytokines and chemokines [60–62], as well as proangiogenic mediators and others involved with the maturation and chemotaxis of dendritic cells [63–65]. Therefore, certain stimuli applied on dentin tissue have a direct repercussion on the subjacent odontoblasts, which may trigger a tissue defense response with intensity directly related to the intensity of the aggression.

The inflammatory response is considered the initial and integral phase of the DPC reparative and regenerative process [1]. According to the degree and persistence of the inflammatory process generated, the tissue response triggered may result in significant pulp damage [56]. Aggressions of light intensity result in an increase in the regulation of dentinogenesis by the subjacent primary odontoblasts (reactionary dentinogenesis). However, when the injury is moderate or intense, resulting in the death of the primary odontoblasts that line the dentin internally, the reactionary condition becomes more complex, involving the recruitment of undifferentiated mesenchymal cells from the pulp and their differentiation into odontoblast-like cells, which begin to synthesize and deposit dentin matrix (reparative dentinogenesis) with an amorphous, sometimes atubular characteristic [66–68]. Consequently, the clinical use of dental materials or

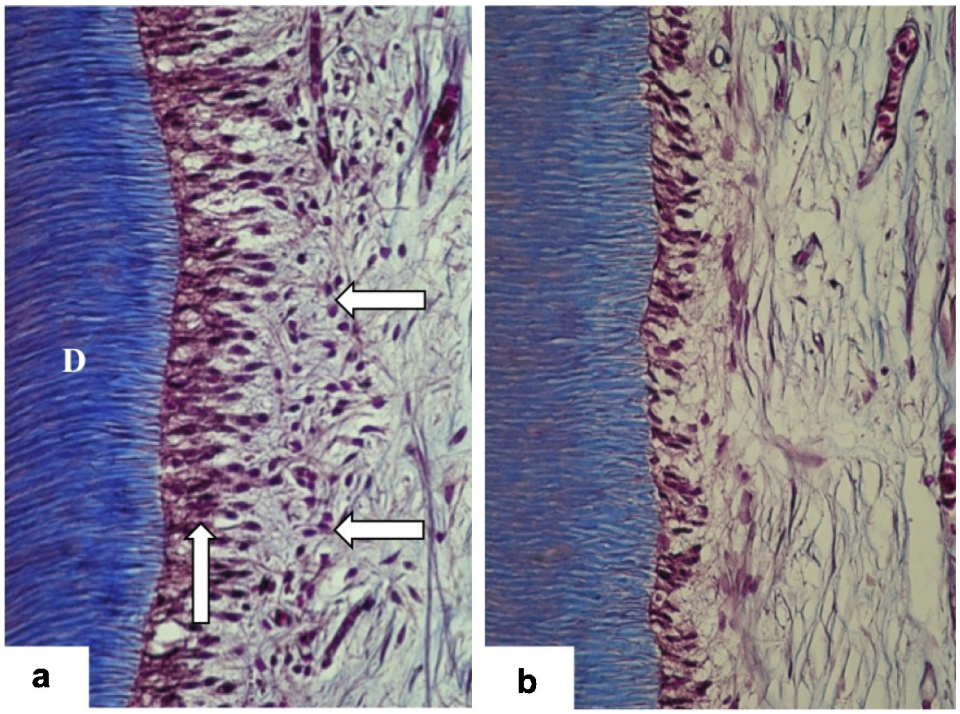

Fig. 4 – (a) Histological section of a young human tooth. Note the morphology of a number of tall odontoblasts (vertical arrow) that are underlying the dentin (D). A number of mesenchymal cells are also observed (arrows); Masson's Trichrome, 250×. (b) Histological section of an old human tooth. Note a reduced number of odontoblasts which are smaller than those observed in Fig. 4a. Only a few mesenchymal cells can be seen; Masson's Trichrome, 250×.

application of operative techniques that result in intense pulp cell death, followed by the deposition of reparative dentin, do not characterize acceptable and safe clinical procedures. This is because, in addition to rupture in the equilibrium of the dentin/pulp connection by the death of the primary odontoblasts, loss of their cytoplasmic prolongations and intra-tubular nerve endings, the recruitment of a large number of undifferentiated mesenchymal cells from the pulp also occurs, resulting in the reduction in cellularity and consequent early aging of the pulp tissue (Fig. 4a and b).

The DPC response to the injuries is not limited to the pulp cells only. Dentin functions as a source of bioactive proteins and growth factors, such as DMP-1, DSPP and TGF-β which, after dentinogenesis, remain trapped in this tubular substrate [55]. Dentin demineralization may occur during the development of a carious process, cavity preparation or even as a result of acid etching or application of certain acidic dental materials on this tissue. Within this context, the bioactive molecules and growth factors released may induce the synthesis, deposition and mineralization of the dentin matrix by the subjacent odontoblasts, and also participate in the process of proliferation and differentiation of other pulp cells [68]. This increase in local cell activity also effectively contributes to the process of obliteration of the dentinal tubules (dentin sclerosis), reducing dentin permeability [66,67]. Therefore, the regulation of odontoblastic activity is a central point for pulp tissue homeostasis, so that knowledge of this process is fundamental for the development of new dental materials and recommendation of the clinical use of new operative procedures, which must preserve the integrity and functions of the DPC.

4. Strategies for improving the biocompatibility of operative techniques and dental materials with the dentin–pulp complex

In view of the knowledge about the DPC responses to aggressor agents, such as bacteria and their products (Fig. 5a–d), diverse strategies have been proposed with the object of repairing and preserving dentin and pulp homeostasis and vitality, particularly when these tissues are challenged to interact with new dental materials. Therefore, the search for dental materials that maintain the integrity and/or stimulate the repair/regeneration of the DPC goes back to around 70 years ago, when calcium hydroxide-based products were used to induce the formation of a mineralized barrier in teeth with exposed pulp tissue [69,70].

Knowledge of the process of development and evolution of caries disease, involving factors related to the risks through to the available treatments, as well as understanding of the DPC behavior against aggressor agents, have promoted significant changes in the techniques for the use and application of the existent dental materials, with a view to minimizing intervention and maximum biocompatibility. Moreover, with the scientific advancements in the areas of biotechnology and diagnosis, Dentistry entered a new era in the development of biomaterials, with the help of tissue engineering using stem cells and nanotechnology. The central aim of studies within these areas is to increase the biocompatibility of dental materials, modulate the DPC response, and guarantee pulp vitality throughout the life of the individual [11,68]. Therefore, the

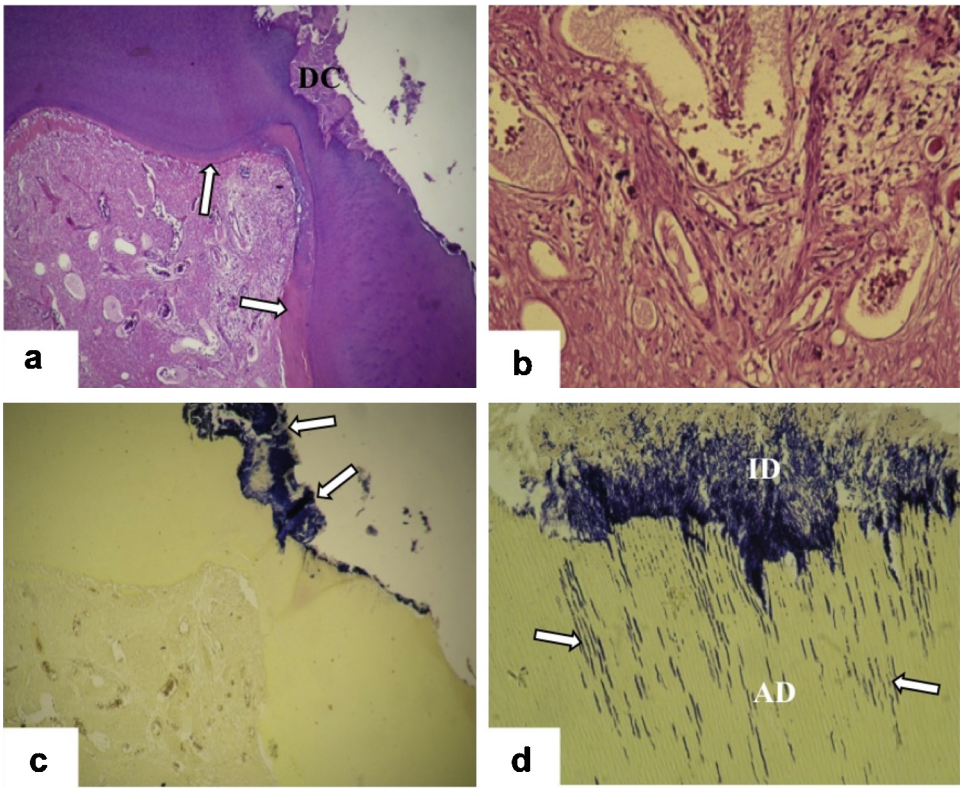

Fig. 5 – (a) Human primary tooth with a deep proximal decay (DC). Note the intense inflammatory pulp response associated with tertiary dentin formation (arrows); H/E, 32×. (b) High magnification of the pulp tissue which exhibits a number of congested and dilated blood vessels among inflammatory cells; H/E, 160×. (c) General view of the microorganisms (arrows) evidenced by a specific gram staining technique; Brown & Brenn, 32×. (d) Detail of the decay on dentin. Note the superficial infected dentin (ID) which is totally disorganized (necrotic). The subjacent affected dentin (AD), which can be remineralized, presents a number of microorganisms inside the dentinal tubules (arrows); Brown & Brenn, 125×.

interactions of dental materials with the DPC, advancements in the development of biomaterials, and the changes in the approach to clinical procedures with the purpose of improving the biocompatibility related to restorative treatment will be described below.

5. Minimal intervention dentistry – MID

With better understanding of caries disease, the therapeutic approach to treatment of this disease has undergone profound changes, originating minimal intervention dentistry (MID). The primary goal of MID is to maintain the tooth in a healthy and functional condition throughout the entire life of the individual, and for this purpose, the following basic principles have been established: Early detection of caries lesions and risk evaluation; (2) remineralization of demineralized enamel and dentin; (3) preventive measures against caries; (4) minimally invasive interventions; and (5) repair instead of replacement of restorations [71,72].

All of these principles aim to reduce the removal and destruction of dental hard tissues, revert the lesions by means of enamel and dentin remineralization, and consequently maintaining pulp homeostasis and vitality throughout the period of life of the tooth. This change in how to treat the disease has arisen from the understanding of caries as a disease of a behavioral nature, with a bacterial component that requires actions involving dietary control and measures for mechanical disorganization of bacterial biofilm by the individual, associated with actions of prevention to be performed by the dental surgeon [72]. Adoption of the MID principles has only been possible with the advancements in the areas of Cariology and Adhesive Dentistry, which recommend the maintenance of caries-affected dentin, stimulation of remineralization of this tissue, and performing conservative cavity preparations capable of maintaining the restorative material in position by bonding it to the hard tissues of the tooth.

6. Partial removal of carious dental tissue

Knowledge of the factors that lead to the development of caries lesions, with the presence of an active biofilm on the mineralized tooth structures, has directed the change in the quantity of contaminated tissue to be removed from cavities. Over the last few decades, diverse clinical studies have been developed, using the methodology of partial carious (infected) tissue removal, and a lining material, such as calcium hydroxide cement, applied on the remaining dentin (affected by caries) [73,74]. These studies have observed the presence of a

harder, darker dentin and a lower quantity of viable microorganisms during the reopening of deep cavities after a period of 6–8 months from the procedure. All these new concepts are integrated into the modern approach to caries, in which it is not necessary to reopen this cavity to evaluate the remineralization of this tissue [75]. It must be taken into consideration that the total removal of carious tissue increases the risk of pulp exposure, and reduces the clinical success rate of the treatment [76]. Therefore, the dentin affected by caries must be maintained and covered with a material capable of inducing the remineralization of this tissue, thereby preventing pulp tissue exposure and consequently increasing the possibility of successful restorative treatment.

7. Materials capable of inducing remineralization

The remineralization of demineralized dentin may occur as a result of the activity of odontoblasts and their prolongations, which provide vital pulp with calcium and phosphate, and also by the diffusion of some ions, such as fluoride, calcium and phosphate, released from the lining materials [77]. The most used dental material for lining caries-affected dentin has been calcium hydroxide cement, considered the gold standard for indirect and direct pulp capping [68]. Various clinical studies have demonstrated the capacity of this material in remineralizing the caries-affected dentin of the cavity floor [73,74,78]. The induction of dentin remineralization by calcium hydroxide cement may be associated with its capacity to extract bioactive molecules from dentin as a result of its alkalinity, and also by the release of calcium ions [68,79]. However, this material has some disadvantages, such as low mechanical strength, high solubility and lack of bonding to dentin.

With dissemination of the MID principles, there has been a search for materials capable of promoting adequate cavity sealing for a long period, and to allow resolution of the inflammatory condition by the DPC itself. Conventional Glass Ionomer Cements (GIC) represent a class of dental materials that maintain the advantages of calcium hydroxide, such as biocompatibility and antimicrobial activity; additionally, these cements present better chemical, physical and mechanical properties [80]. The GIC capacity of releasing fluoride and adhere to hard dental tissues has made ionomer cement the material of choice to protect the DPC [81]. The association of water-soluble polyacrylic acid and ion leachable glass with organic monomers, associated with an initiator system has allowed the development of resin-modified glass ionomer cements (RMGICs). These ionomer resin cements have higher flexural, diametral tensile strength and modulus of elasticity when compared with conventional GICs, in addition to having the capacity of copolymerizing with resin restorative materials [82]. Recently, Costa et al. [83] demonstrated that lining very deep cavities with two different formulations of one and the same RMGIC caused a slight pulp inflammatory reaction. This initial tissue response resolved itself over time, indicating the biocompatibility of this type of light polymerizing cement. Mickenautsch et al. [84], conducted a review of the literature, seeking to make a comparative approach to pulp response induced by RMGICs and calcium hydroxide. Considering pulp inflammatory response, hard tissue repair, microleakage and changes in the odontoblast layer, the authors determined that there was no significant difference between the studied materials. The capacity of glass ionomer cements to release fluoride and calcium, leads to the absorption of these ions by the subjacent dentin, resulting in the remineralization of this tissue, and inhibiting caries progression [85,86].

Although dentin is capable of being remineralized, this dental tissue presents a larger organic content when compared with enamel, and is characterized as a complex substrate, since its remineralization depends on the existence of residual inorganic crystals. For this reason, the effects of fluorides on enamel are more evident when compared with dentin under the same experimental conditions [87]. Nevertheless, remineralization of this tubular tissue determines the integrity and resistance of the remaining dentin between the cavity floor and the subjacent pulp, which is imperative for the success of restorative treatment and maintenance of the DPC homeostasis. Therefore, biomimetic strategies have arisen for dentin substrate biomineralization. These strategies use collagen itself as a scaffold for the deposition of minerals in the presence of non collagenous proteins [88–90], which act as nucleators or even inhibitors of mineralization by means of their functional groups, which are capable of promoting collagen phosphorylation and inducing biomineralization. Therefore, these strategies seek to use compounds that have the potential to promote the phosphorylation of collagen, simulating the action of extracellular matrix proteins associated with composites, which enable the formation of calcium phosphate precursors, allowing the local growth of the inorganic crystal.

Zhang et al. [90] observed that the phosphorylation of Collagen Type I on the demineralized dentin surface, using sodium trimetaphosphate (STMP) associated with pre-treatment with a saturated calcium hydroxide solution for this purpose, resulted in an increase in the biomineralization of artificial caries lesions, when compared with the group in which the lesions were submitted to treatment with fluorides only. According to the authors, phosphorylation of collagen promotes alterations on the dentin surface, making it more negatively charged and with less interfacial free energy between the substrate and medium, conditions necessary for nucleation of the crystals. Other researchers have associated STMP with a remineralization solution containing components of Portland cement and polyacrylic acid [88]. The acid agent was used to stabilize the amorphous calcium phosphate as a liquid nano-precursor component to penetrate into the intrafibrillar compartments of collagen. This method was shown to be promising in the remineralization of artificial caries lesions, so that the authors began to indicate the incorporation of these components into demineralized dentin before the application of adhesive systems [88]. More recently, the addition of STMP and polyacrylic acid to MTA cement powder resulted in greater remineralization of artificial caries lesions, and it was possible to observe dentinal tubules obliteration with apatite crystals [89]. These *in vitro* studies, using agents that mimic the action of dentin extracellular matrix proteins, with the aim of inducing the biomineralization of this tubular dental tissue, characterizes a promising strategy that may favor the biocompatibility of esthetic dental

restorations. Nevertheless, clinical studies are necessary to evaluate whether this therapy is able to results in completely successful treatment, which should be characterized by esthetic and functional success of the restoration associated with the maintenance of the homeostasis, vitality and functions of the dentin–pulp complex.

8. Adhesive restorative dentistry

As previously described, it was the development of adhesive resin materials that provided the present advancement of MID, which recommends the minimal removal of tooth tissue, restricted to tissue that cannot be remineralized, and good sealing of the tooth cavity [72,75]. Adhesive resin cements and bonding agents may be applied for sealing pits and fissures as well as for direct and indirect cavity restorations. In spite of the broad indication for clinical use, these resin-based dental materials are known for the toxicity of the residual resin monomers they release [91]. These components coming from such dental materials are capable of causing oxidative stress in the cells, cell death by apoptosis, inducing genotoxic effects and generating delay in the cell cycle [91]. In addition, when they reach the pulp tissue *via* the dentinal tubules, the monomers may inhibit the function of odontoblasts, reducing the production of proteins related to dentinal matrix deposition, and retard odontogenic differentiation and the mineralization process. Therefore, care must be taken when resin dental materials are clinically used in vital teeth, particularly when working on deep dentin substrate or exposed pulp tissue. Approximately one decade ago, de Souza Costa et al. [92] evaluated the pulp response after acid etching of dentin and application of adhesive systems in deep cavities prepared in human premolars. The histological cuts analyzed revealed disruption of the odontoblastic layer related to the cavity floor. The authors also observed the recruitment of inflammatory cells in the midst of dilated and congested blood vessels and disorganization of the adjacent pulp tissue. Within this context it was related that the remaining dentin between the cavity floor and pulp, with a thickness of less than 300 μm was not capable of preventing pulp damage when adhesive agents are applied on dentin previously etched with acid (Fig. 6a and b). From these results and other scientific data also obtained by means of researches developed in human teeth [6,93], some strategies began to be evaluated with the aim of increasing the biocompatibility of adhesive systems. These strategies range from the use of dentin remineralization agents before the application of resin materials, through to the clinical use of a new modality of self-etching adhesive agent. Although adhesive systems that use prior acid etching are frequently used in dental offices, their technique is extremely sensitive and their efficacy depends on diverse factors, such as the degree of dentin humidity. Therefore, the self-etching adhesive systems have been indicated because they promote superficial demineralization of dentin, forming a thinner and more homogeneous hybrid [94]. Non removal of the smear layer and smear plug associated with less thickness of the hybrid layer appears to limit the toxicity of self-etching adhesive systems on the pulp. In 2007, de Souza Costa et al. [93] evaluated the biocompatibility of a RMGIC (Vitrebond®) and a self-etching adhesive system (Clearfil SE Bond) applied in deep cavities. The authors observed that in the majority of the specimens in which the adhesive system was applied, the pulp exhibited a discrete inflammatory response after 7 days. This pulp inflammatory reaction was maintained over time in some specimens, in which the remaining dentin thickness was very thin. On the other hand, the RMGIC was considered biocompatible, irrespective of the tooth cavity depth (Fig. 7a and b). In a current study, our research group applied a RMGIC (Vitremer®) on deep dentin pre-treated with a primer (acrylic acid + HEMA). This therapy caused and intense pulp damage associated with inner dentin resorption (Fig. 7c and d). We suggested that the pre-treatment of the dentin substrate with an acidic solution containing a monomer before filling the cavity with Vitremer® caused the intense pulp damage. One must not forget that the amount per area and diameter of the dentinal tubules increase as one comes closer to the pulp. Therefore, the intense exudation of dentinal fluid, leading to the elevated humidity of deep dentin interferes in the polymerization process of adhesive systems, which results in the maintenance of a large quantity of highly toxic free residual monomers at the site [95]. Associated with this condition, the reduced area of intertubular dentin associated with the pressure of dentinal fluid exudation, does not favor the formation of a homogeneous and stable hybrid layer. All of these factors in conjunction negatively influence the process of dentinal tubule sealing and resin material bond to deep dentin. It has been demonstrated that during the polymerization of adhesive systems, there is an influx of fluid through the dentinal tubules, and this phenomenon favors the diffusion of free residual monomers in the direction of the pulp tissue [96]. When these toxic resin components reach the pulp, they are capable of triggering a local inflammatory response [91,97], which varies in intensity according to the quantity of product in contact with this specialized connective tissue. As has been demonstrated by de Souza Costa et al. [92,93], there is less pulp damage caused by self-etching adhesive systems than that induced by the conventional adhesive systems, which require acid etching before they are applied on dentin. Therefore, although it has never been demonstrated that acid agents in gel are capable of reaching the pulp, even when applied on very deep dentin in vital teeth, it appears that these hypertonic products not only enlarge the internal diameter of dentinal tubules, but also favors rapid exudation of dentinal fluid, thus increasing the humidity of the local dentin. Certainly, these effects of the acid agents play a fundamental role in the transdentinal diffusion of toxic monomers and consequently on the pulp damage observed when this adhesive restorative technique is applied in very deep cavities [41].

9. Pulp exposure

Once the structural integrity of dentin has been ruptured, whether by a caries process or by trauma, the exposed pulp with the potential to repair and reverse the damage undergone, must be submitted to a specific therapy what allows for the reestablishment of vitality and function of this specialized conjunctive tissue [68]. Therefore, the success of Vital

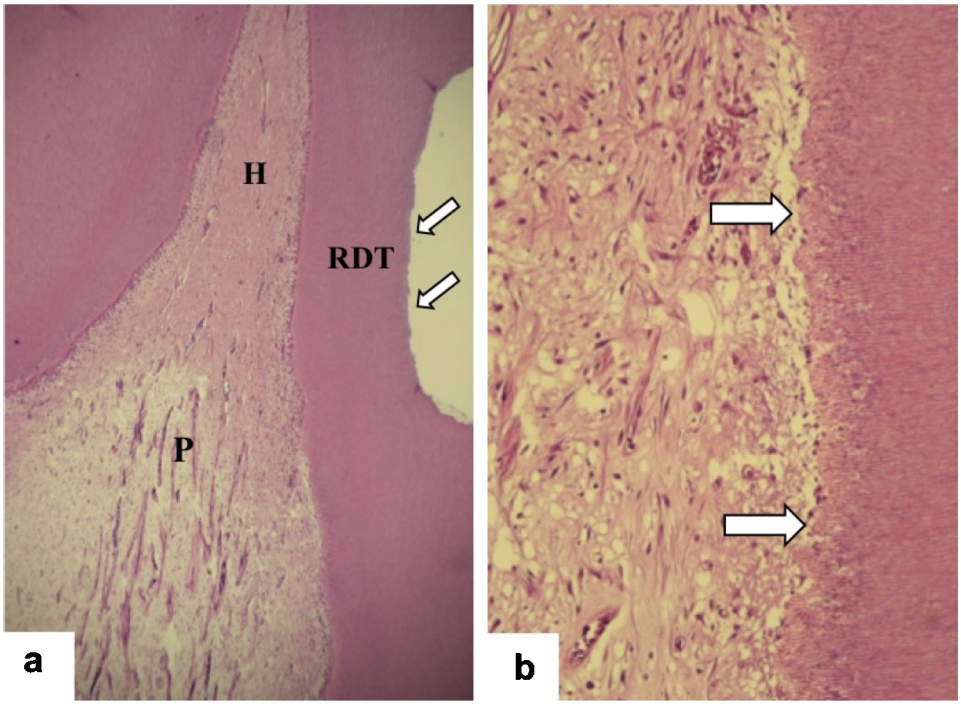

Fig. 6 – (a) Deep cavity prepared in a sound human premolar of a young patient. After conditioning the cavity walls with acid agent, an adhesive system was applied on dentin and the cavity was restored with resin composite. Note the thin remaining dentin thickness (RDT) between the cavity floor (arrows) and the subjacent pulp tissue (P); H/E, 32×. (b) Detail of the superficial pulp area related to the cavity floor. Despite the complete disruption of the odontoblast layer (arrows) and the hyaline alteration (H) observed in the pulp horn, only a mild inflammatory pulp response occurred at short-term period of evaluation; H/E, 125×.

Pulp Therapy depends directly on careful clinical diagnosis of the pulp condition, and may be indicated for teeth that present an asymptomatic condition or one of provoked pain, normal response to the percussion test, normal radiographic aspects, possibility of achieving homeostasis of pulp bleeding and normal characteristics of pulp tissue color and consistency [98]. A recent systematic review of the literature evaluated the percentage of success when conservative pulp therapy was applied in vital permanent teeth, however, with pulp exposure during the mechanical removal of carious dentin. The authors observed that this success rate ranged from 72.9 to 99.4% when clinical evaluation of the pulp condition was correctly performed [98].

The different formulations of calcium hydroxide have been extensively studied as a direct pulp capping agent, as this product has been used as the gold standard to compare pulp response to new dental materials developed for direct application on exposed pulps [92,6,93]. The biologic action of calcium hydroxide on pulp appears to be associated with its solubilization and consequent increase in the local pH, since this caustic potential of the product results in superficial necrosis of the pulp tissue [99]. But at present it has been suggested that the alkalinity of calcium hydroxide causes dissolution of the dentin substrate adjacent to the pulp wound, leading to the release of bioactive molecules trapped in this mineralized tissue [79]. When in turn, these molecules are available on the pulp wound they will act directly in recruiting mesenchymal cells for the periphery of the pulp in the differentiation of odontoblast-like cells, which begin to synthesize and deposit reparative dentin matrix at the site. However, some of the deficiencies of calcium hydroxide, such as the presence of defects in the form of tunnels in the mineralized barrier formed after use of the material, and the lack of bond of this material to dentin have been suggested as causes of failure in this type of therapy [1].

Mineral trioxide aggregate (MTA) was introduced on the market for filling the root canal after endodontic treatment and for sealing accidental perforations [100,101]. Due to its biocompatibility, capacity of inducing reparative dentin formation in animals, and limited toxic effect on different cell types, including odontoblast-like cells [102], this cement began to be recommended for direct pulp capping as well [103]. The mechanism of action of MTA is similar to that of calcium hydroxide, since this is the main component released after the product sets in contact with pulp tissue [104]. Initially, MTA in contact with the pulp causes a thin layer of coagulation necrosis, which is slowly replaced by a dystrophic calcification added to the presence of dentin matrix produced by recently differentiated odontoblast-like cells. Nevertheless, it is known that MTA presents some disadvantages, such as a long setting time (3–4 h), it is difficult to manipulate, and high cost [103]. In a recent evaluation performed by our research group, ProRoot MTA was applied on the pulps of mechanically exposed human pulps. In this study it was observed that the components of MTA diffused through the pulp tissue, and 60 days after the capping procedure, these components were found

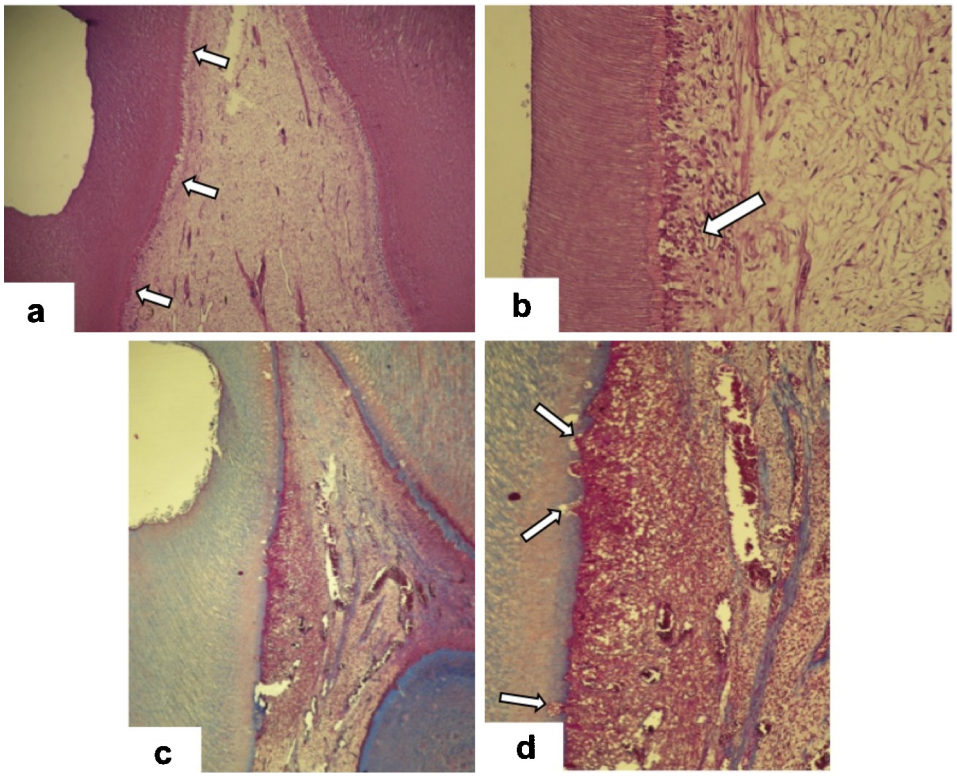

Fig. 7 – (a) Deep cavity prepared in a sound human premolar of a young patient. Before performing the adhesive restoration of the dental cavity, the RMGIC Vitrebond© was applied on the cavity floor. Note the continuous odontoblast layer (arrows); H/E, 42×. (b) In this human premolar in which Vitrebond© was also used as liner, only a mild inflammatory pulp response was observed (arrow); H/E, 125×. (c) In this sound young premolar, the cavity floor was treated with an acidic primer and the RMGIC Vitremer© was used as liner. Then, the cavity was restored with adhesive system and composite resin. Note the intense pulp response; Masson's Trichrome 32×. (d) High magnification of Fig. 7c, in which the pulp tissue related to the cavity floor can be seen. Note the intense inflammatory pulp response associated with inner dentin resorption (arrows); Masson's Trichrome 125×.

in endothelial cells and inside of blood vessels of the pulp (Fig. 8a–d).

More recently, other biomaterials indicated for capping exposed pulps have been the target of researches, with tricalcium silicate cement, one of the main components of MTA, being shown to be outstanding [105,106]. This cement, commercially available under the name of Biodentine, is composed of tricalcium silicate powder, calcium carbonate and a radiopaque agent [107]. Due to its similarity to MTA, its mechanism of action is also based on the release of calcium hydroxide and hydrated calcium silicate, increasing the pH at the pulp wound site [107]. In 2011, Peng et al. [105] evaluated the effects of tricalcium silicate in a pulp cell culture and observed that this material induced cell proliferation compared to calcium hydroxide. In addition, the authors demonstrated that the material caused an increase in gene expression of proteins directly related to dentin matrix deposition, such as alkaline phosphatase and dentin matrix protein 1 (DMP1), as well as greater formation of mineralized nodules. The potential of this material to promote pulp tissue repair in rat molars has also been related with the formation of a mineralized barrier 30 days after the capping procedure [108]. According to the manufacturer, Biodentine presents a reduced setting time (around 12 min), easy manipulation (powder and liquid) and bond to dental tissues. Furthermore, the zone of necrosis generated by the product is smaller than that caused by calcium hydroxide, since its components are released only during setting of the material, and also because it is less soluble than calcium hydroxide. However, there is not sufficient scientific evidence yet, determining the safety of clinical application of Biodentine on the exposed pulps of human teeth.

10. Future perspectives for biomaterials

Nanotechnology is an applied science that controls the structure and properties of materials at atomic and molecular levels [97]. The so-called nanoparticles have an ample free surface for interaction with the medium, and therefore they are capable of forming strong bonds with other materials and with one another. These characteristics have resulted in a diversity of applications of nanotechnology in dentistry, from improving the physical properties of composites through to inserting antibacterial components in resin composites [97]. The addition of nanoparticles containing quaternary ammonia in composites generated an antimicrobial effect on contact

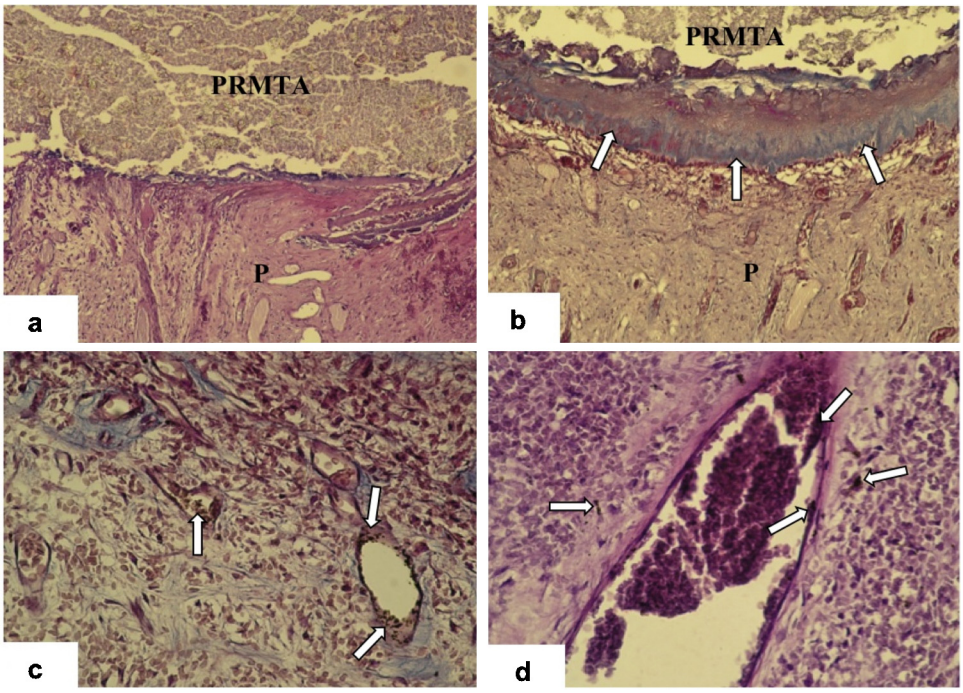

Fig. 8 – (a) Sound human premolar subjected to pulp exposure. Pro-Root MTA (PRMTA) was applied on the pulpal wound (P) and the tooth extracted 7 days after the operative procedure; Masson's Trichrome, 64×. (b) Sixty days after pulp capping with Pro-Root MTA, the tooth was extracted and submitted to laboratorial process. Note the complete deposition of a hard barrier (arrows) between the capping agent and the subjacent pulp tissue; Masson's Trichrome, 64×. (c) Detail of the pulp tissue on which Pro-Root MTA was applied for 60 days. Note that components released from the capping agent are observed in the extracellular matrix and in a blood vessel (arrows); Masson's Trichrome, 125×. (d) High magnification of a pulp blood vessel. Components of MTA spread inside and outside of a large blood vessel located at the central part of the pulp issues are observed (arrows); Masson's Trichrome, 250×.

with *S. mutans*, which lasted for one month, without changing the mechanical properties of this resin material [109]. Another example of the application of nanotechnology in Dentistry is the process of remineralizing initial caries lesions by means of casein phosphopeptide-amorphous calcium phosphate nanocomplexes, which may be used as a vehicle for bioavailable calcium and phosphate ions [110]. The insertion of nano-hydroxyapatite and nanoparticles of calcium carbonate in dentifrices has also been shown to be effective in the process of remineralizing initial caries lesions *in vitro* [111,112]. Both of the nanoparticles act as reservoirs of calcium and phosphate, helping to maintain a level of supersaturation of these ions in the surrounding fluid [113]. All of these strategies seek to reduce the loss of dental structure, preventing the evolution of a clinical condition to a situation of irreversible damage to the DPC.

Another strategy that has being widely evaluated for several years is based on the application of tissue engineering knowledge associated with stem cells for regeneration of the DPC [114,115]. In spite of having been isolated from dental pulp and periapical tissue, stem cells continue to be studied in order to investigate their specificity, potentiality and applicability in tissue engineering for the reconstruction of pulp and dentin [1].

It has been demonstrated that tissue engineering associated with the use of stem cells may be indicated for the treatment of exposed vital pulp situations, and for non-vital teeth, creating the possibility of developing a new pulp tissue capable of replacing the lost pulp. This strategy was proposed due to the potential of stem cells from the pulp of primary and permanent teeth to differentiate into odontoblasts *in vitro*, with the capacity to produce tubular dentin *in vivo* [114,116]. A recent *in vivo* study was developed, using stem cells from pig primary teeth associated with a beta-phosphate-tricalcium scaffold to repair defects in the pulp chamber floor of pig premolars [117]. After 16 weeks, the authors observed complete closure of the defect by a mineralized barrier, both in histological cuts and by tomography, and concluded that the association of stem cells with the scaffold resulted in adequate dentin regeneration [117].

For devitalized teeth, biological strategies have also been proposed to reduce the number of failures resulting from endodontic treatment, which is still based on disinfection of root canal systems and sealing them with an inert material [1]. In the last decade, disinfection of root canals with antibiotics and formation of a blood clot inside the canal by inducing hemorrhage in the periapical region has been proposed. This therapy, known as revascularization, has not been recognized as a technique for pulp tissue regeneration, because up to now, there has been no scientific proof that the conjunctive tissue formed from the periapical site specializes in a pulp tissue capable of depositing and mineralizing dentin matrix

[1]. Nevertheless, root canal system filling with a specialized connective tissue, or not, appears to be an efficient alternative in the prevention of future infectious processes. Apart from this alternative, filling the root canal with a scaffold of peptide nanofibers associated with stem cells was recently presented as an alternative for pulp tissue regeneration [115]. The authors observed that 35 days after filling the root canal, there was the formation of a new pulp tissue occupying the entire root canal, with the presence of proliferative cells and blood vessels. This new tissue formed was comparable with the pulp tissue of a young tooth [115].

In spite of the intense advancements in gene therapy, in Dentistry there are still many questions without clear answers that characterize the efficiency of the laboratory procedures used to maintain or restore pulp vitality, when compared with conventional treatments. The sensitivity and reproducibility of tissue engineering techniques applied to repair/regeneration of the dentin–pulp complex, the equipment and structures required to convert this approach into one capable of being performed in office, is also a great challenge [1]. Therefore, in spite of characterizing a promising therapy, the pathway to its clinical applicability is long, and it probably will not be feasible for diverse specific clinical conditions.

REFERENCES

[1] Simon SR, Berdal A, Cooper PR, Lumley PJ, Tomson PL, Smith AJ. Dentin–pulp complex regeneration: from lab to clinic. Adv Dent Res 2011;23:340–5.

[2] Smith AJ, Scheven BA, Takahashi Y, Ferracane JL, Shelton RM, Cooper PR. Dentine as a bioactive extracellular matrix. Arch Oral Biol 2012;57:109–21.

[3] Cox CF, Hafez AA, Akimoto M, Otsuki M, Suzuki S, Tarim B. Biocompatibility of primer, adhesive and resin composite system on non-exposed and exposed pulps of non-human primate teeth. Am J Dent 1998;10: S55–63.

[4] Kitasako Y, Inokoshi S, Tagami J. Effects of direct resin pulp capping technique on short-term response of mechanically exposed pulps. J Dent 1999;27:257–63.

[5] Accorinte MLR, Loguercio AD, Reis A, Muench A, Araújo VC. Adverse effects of human pulps after direct pulp capping with the different components from a total-etch, three-step adhesive system. Dent Mater 2005;21: 599–607.

[6] de Souza Costa CA, Hebling J, Randall RC. Human pulp response to resin cements used to bond inlay restorations. Dent Mater 2006;22:954–62.

[7] Nowicka A, Parafiniuk M, Lipski M, Lichota D, Buczkowska-Radlinska J. Pulpo-dentin complex response after direct capping with self-etch adhesive systems. Folia Histochem Cytobiol 2012;50:565–73.

[8] Markowitz K. Pretty painful: why does tooth bleaching hurt? Med Hypotheses 2010;8(74):5–840.

[9] Reis A, Tay LY, Herrera DR, Kossatz S, Loguercio AD. Clinical effects of prolonged application time of an in-office bleaching gel. Oper Dent 2011;36:590–6.

[10] Tay LY, Kose C, Herrera DR, Reis A, Loguercio AD. Long-term efficacy of in-office and at-home bleaching: a 2-year double-blind randomized clinical trial. Am J Dent 2012;25:199–204.

[11] Wataha JC. Predicting clinical biological responses to dental materials. Dent Mater 2012;28:23–40.

[12] Autian J. The use of rabbit implants and tissue culture tests for the evaluation of dental materials. Int Dent J 1970;20:481–90.

[13] Bayne SC. Correlation of clinical performance with 'in vitro tests' of restorative dental materials that use polymer-based matrices. Dent Mater 2012;28:52–71.

[14] Lanza CR, de Souza Costa CA, Furlan M, Alécio A, Hebling J. Transdentinal diffusion and cytotoxicity of self-etching adhesive systems. Cell Biol Toxicol 2009;25:533–43.

[15] Soares DG, Basso FG, Hebling J, de Souza Costa CA. Concentrations of and application protocols for hydrogen peroxide bleaching gels: effects on pulp cell viability and whitening efficacy. J Dent 2014;42:185–98.

[16] Bianchi L, Ribeiro AP, de Oliveira Carrilho MR, Pashley DH, de Souza Costa CA, Hebling J. Transdentinal cytotoxicity of experimental adhesive systems of different hydrophilicity applied to ethanol-saturated dentin. Dent Mater 2013;29:980–90.

[17] Grayson WL, Martens TP, Eng GM, Radisic M, Vunjak-Novakovic G. Biomimetic approach to tissue engineering. Semin Cell Dev Biol 2009;20:665–73.

[18] Hume WR. A new technique for screening chemical toxicity to the pulp from dental restorative materials and procedures. J Dent Res 1985;64:1322–5.

[19] Hanks CT, Wataha JC, Parsell RR, Strawn SE, Fat JC. Permeability of biological and synthetic molecules through dentine. J Oral Rehabil 1994;21:475–87.

[20] Hanks CT, Fat JC, Wataha JC, Corcoran JF. Cytotoxicity and dentin permeability of carbamide peroxide and hydrogen peroxide vital bleaching materials in vitro. J Dent Res 1993;72:931–8.

[21] Modena KC, Casas-Apayco LC, Atta MT, Costa CA, Hebling J, Sipert CR, et al. Cytotoxicity and biocompatibility of direct and indirect pulp capping materials. J Appl Oral Sci 2009;17:544–54.

[22] Pashley DH. Consideration of dentine permeability in cytotoxicity testing. Int Endod J 1988;21:143–54.

[23] Galler K, Hiller K, Ettl T, Schmalz G. Selective influence of dentin thickness upon cytotoxicity of dentin contacting materials. J Endod 2005;31:396–9.

[24] Schmalz G, Schuster U, Nuetzel K, Schweikl H. An in vitro pulp chamber with three-dimensional cell-cultures. J Endod 1999;25:24–9.

[25] Trindade FZ, Ribeiro AP, Sacono NT, Oliveira CF, Lessa FC, Hebling J, et al. Trans-enamel and trans-dentinal cytotoxic effects of a 35% H_2O_2 bleaching gel on cultured odontoblast cell lines after consecutive applications. Int Endod J 2009;42:516–24.

[26] Soares DG, Ribeiro AP, Sacono NT, Coldebella CR, Hebling J, Costa CAS. Transenamel and transdentinal cytotoxicity of carbamide peroxide bleaching gels on odontoblast-like MDPC-23 cells. Int Endod J 2011;44:116–25.

[27] Hanks CT, Fang D, Sun Z, Edwards CA, Butler WT. Dentin-specific proteins in the MDPC-23 cell line. Eur J Oral Sci 1998;106:S60–6.

[28] Semeghini MS, Fernandes RR, Chimello DT, Oliveira FS, Bombonato-Prado KF. In vitro evaluation of the odontogenic potential of mouse undifferentiated pulp cells. Braz Dent J 2012;23:328–36.

[29] Arenholt-Binslev D, Bleeg H. Characterization of two types of oral fibroblasts with a potential application to cellular toxicity studies: tooth pulp fibroblasts and buccal mucosa fibroblasts. Int Endod J 1990;23:84–91.

[30] Huang GJ, Shagramanova K, Chan SW. Formation of odontoblast-like cells from cultured human dental pulp cells on dentin in vitro. J Endod 2006;32:1066–73.

[31] Murray PE, García-Godoy C, García-Godoy F. How is he biocompatibility of dental biomaterials evaluated? Med Oral Patol Oral Cir Bucal 2007;12:E258–66.
[32] Galler KM, D'Souza RN, Hartgerink JD, Schmalz G. Scaffolds for dental pulp tissue engineering. Adv Dent Res 2011;23:333–9.
[33] Wiegand C, Hipler U. Methods for the measurement of cell and tissue compatibility including tissue regeneration process. GMS Krankenhaushyg Interdiszip 2008;11:1–9.
[34] Okada Y, Maeno E, Mori S. Anion channel involved in induction of apoptosis and necrosis. Adv Exp Med Biol 2004;559:205–9.
[35] Sato C, Rodrigues FA, Garcia DM, Vidal CPM, Pashley DH, T.Jaderhane L, et al. Tooth bleaching increases dentinal protease activity. J Dent Res 2013;92:187–92.
[36] Nakade O, Koyama H, Arai J, Ariji H, Takada J, Kaku T. Stimulation by low concentrations of fluoride of the proliferation and alkaline phosphatase activity of human dental pulp cells in vitro. Arch Oral Biol 1999;44:89–92.
[37] Chang Y, Yang S, Huang F, Liu C, Tai K, Hsieh Y. Proinflammatory cytokines induce cyclooxygenase-2 mRNA and protein expression in human pulp cell cultures. J Endod 2003;29:201–4.
[38] White WJ. The use of laboratory animals in toxicologic research. In: Hayes AW, editor. Principles and methods of toxicology. Taylor & Francis; 2001. p. 773–818.
[39] Meyer O. Testing and assessment strategies, including alternative and new approaches. Toxicol Lett 2003;140:21–30.
[40] Anderson JM. Biological responses to materials. Annu Rev Mater Sci 2001;31:81–110.
[41] Costa CAS, Hebling J, Hanks CT. Current status of pulp capping with dentin adhesive systems: a review. Dent Mater 2000;16:188–97.
[42] Mjör IA. Minimum requirement for new dental materials. J Oral Rehabil 2007;34:907–12.
[43] Bayne SC. Dental restoration for oral rehabilitation-testing of laboratory properties versus clinical performance for clinical decision making. J Oral Rehabil 2007;34:921–32.
[44] Schmalz G. Determination of biocompatibility. In: Schmalz G, Arenholt-Bindslev D, editors. Biocompatibility of dental materials. Springer; 2010. p. 13–40.
[45] Wataha JC. Principles of biocompatibility for dental practitioners. J Prosthet Dent 2001;86:203–9.
[46] Cochrane Collaboration Website: http://www.cochrane.org
[47] Wataha JC, Hanks CT. Biocompatibility testing – what can we anticipate? Trans Acad Dent Mater 1997;10:109–20.
[48] Hanks CT, Wataha JC, Sun Z. In vitro models of biocompatibility: a review. Dent Mater 1996;12:186–93.
[49] Schuster GS, Lefebvre CA, Wataha JC, White SN. Biocompatibility of posterior restorative materials. J Calif Dent Assoc 1996;24:17–31.
[50] Schmalz G. Modern concepts in biocompatibility testing of restorative materials. Trans Acad Dent Mater 1996;9:170–9.
[51] Langeland K. Correlation of screening tests to usage tests. J Endod 1978;4:300–3.
[52] Wennberg A, Mjör IA, Hensten-Pettersen A. Biological evaluation of dental restorative materials: a comparison of different test methods. J Biomed Mater Res 1983;17:23–36.
[53] Pashley D. Pulpodentin complex. In: Hargreaves KM, Goodis HE, editors. Seltzer and Bender's dental pulp. Quintessence; 2002. p. 63–94.
[54] Goldberg M, Kulkarni AB, Young M, Boskey A. Dentin: structure, composition and mineralization. Front Biosci 2011;1:711–35.
[55] Goldberg M, Lacerda-Pinheiro S, Jegat N, Six N, Septier D, Priam F, et al. The impact of bioactive molecules to stimulate tooth repair and regeneration as part of restorative dentistry. Dent Clin North AM 2006;50:277–98.
[56] Cooper PR, Takahashi Y, Graham LW, Simon S, Imazato S, Smith AJ. Inflammation–regeneration interplay in the dentine–pulp complex. J Dent 2010;38:687–97.
[57] Couve E, Osorio R, Schmachtenberg O. The amazing odontoblast: activity, autophagy, and aging. J Dent Res 2013;92:765–72.
[58] Hebling J, Giro EM, Costa CA. Human pulp response after an adhesive system application in deep cavities. J Dent 1999;27:557–64.
[59] Tjärderhane L, Carrilho MR, Breschi L, Tay FR, Pashley DH. Dentin basic structure and composition—an overview. Endod Top 2012;20:3–29.
[60] Farges JC, Keller JF, Carrouel F, Durand SH, Romeas A, Bleicher F, et al. Odontoblasts in the dental pulp immune response. J Exp Zool B Mol Dev Evol 2009;312B:425–36.
[61] Hahn CL, Liewehr FR. Innate immune responses of the dental pulp to caries. J Endod 2007;33:643–51.
[62] Hahn CL, Liewehr FR. Update on the adaptive immune responses of the dental pulp. J Endod 2007;33:773–81.
[63] Telles PD, Hanks CT, Machado MA, Nör JE. Lipoteichoic acid up-regulates VEGF expression in macrophages and pulp cells. J Dent Res 2003;82:466–70.
[64] Schwarze SR, Luo J, Isaacs WB, Jarrard DF. Modulation of CXCL14 (BRAK) expression in prostate cancer. Prostate 2005;64:67–74.
[65] Carmody RJ, Chen YH. Nuclear factor-kappaB: activation and regulation during toll-like receptor signaling. Cell Mol Immunol 2007;4:31–41.
[66] Linde A, Goldberg M. Dentinogenesis. Crit Rev Oral Biol Med 1993;4:679–728.
[67] Smith AJ, Lesot H. Induction and regulation of crown dentinogenesis: embryonic events as a template for dental tissue repair. Crit Rev Oral Biol Med 2001;12:425–37.
[68] Ferracane JL, Cooper PR, Smith AJ. Can interaction of materials with the dentin–pulp complex contribute to dentin regeneration? Odontology 2010;98:2–14.
[69] Zander HA. Reaction of the pulp to calcium hydroxide. J Dent Res 1939;18:373–9.
[70] Zander HA, Glass RL. The healing of phenolized pulp exposures. Oral Surg Oral Med Oral Pathol 1949;2:803–10.
[71] Tyas MJ, Anusavice KJ, Frencken JE, Mount GJ. Minimal intervention dentistry – a review. FDI commission project 1-97. Int Dent J 2000;50:1–12.
[72] Frencken JE, Peters MC, Manton DJ, Leal SC, Gordan VV, Eden E. Minimal intervention dentistry for managing dental caries – a review: report of a FDI task group. Int Dent J 2012;62:223–43.
[73] Bjørndal L, Larsen T. Changes in the cultivable flora in deep carious lesions following a stepwise excavation procedure. Caries Res 2000;34:502–8.
[74] Maltz M, de Oliveira EF, Fontanella V, Bianchi R. A clinical, microbiologic, and radiographic study of deep caries lesions after incomplete caries removal. Quintessence Int 2002;33:151–9.
[75] Kidd EAM. How 'clean' must a cavity be before restoration? Caries Res 2004;38:305–13.
[76] Thompson V, Craig RG, Curro FA, Green WS, Ship JA. Treatment of deep carious lesions by complete excavation or partial removal: a critical review. J Am Dent Assoc 2008;139:705–12.
[77] Peters MC, Bresciani E, Barata TJ, Fagundes TC, Navarro RL, Navarro MF, et al. In vivo dentin remineralization by calcium-phosphate cement. J Dent Res 2010;89:286–91.
[78] Bjørndal L, Thylstrup A. A practice-based study of stepwise excavation of deep carious lesions in permanent teeth: a

1-year follow-up study. Community Dent Oral Epidemiol 1998;26:122–8.
[79] Graham L, Cooper PR, Cassidy N, Nor JE, Sloan AJ, Smith AJ. The effect of calcium hydroxide on solubilisation of bio-active dentine matrix components. Biomaterials 2006;27:2865–73.
[80] Vaderhobli RM. Advances in dental materials. Dent Clin North Am 2011;55:619–25.
[81] Walsh Lj Brostek A. Minimum intervention dentistry principles and objectives. Aust Dent J 2013;58:3–16.
[82] Xie D, Brantley BM, Culbertson G, Wang G. Mechanical properties and microstructures of glass-ionomer cements. Dent Mater 2000;16:129–38.
[83] Costa CAS, Ribeiro AP, Giro EM, Randall RC, Hebling J. Pulp response after application of two resin modified glass ionomer cements (RMGICs) in deep cavities of prepared human teeth. Dent Mater 2011;27:e158–70.
[84] Mickenautsch S, Yengopal V, Banerjee A. Pulp response to resin-modified glass ionomer and calcium hydroxide cements in deep cavities: a quantitative systematic review. Dent Mater 2010;26:761–70.
[85] Forsten L. Fluoride release and uptake by glass-ionomers and related materials and its clinical effect. Biomaterials 1998;19:503–8.
[86] Ngo HC, Mount G, Mc Intyre J, Tuisuva J, Von Doussa RJ. Chemical exchange between glass-ionomer restorations and residual carious dentine in permanent molars: an in vivo study. J Dent 2006;34:608–13.
[87] Fan Y, Sun Z, Moradian-Oldak J. Controlled remineralization of enamel in the presence of amelogenin and fluoride. Biomaterials 2009;30:478–83.
[88] Liu Y, Li N, Qi Y, Niu LN, Elshafiy S, Mao J, et al. The use of sodium trimetaphosphate as a biomimetic analog of matrix phosphoproteins for remineralization of artificial caries-like dentin. Dent Mater 2011;27:465–77.
[89] Qi YP, Li N, Niu LN, Primus CM, Ling JQ, Pashley DH, et al. Remineralization of artificial dentinal caries lesions by biomimetically modified mineral trioxide aggregate. Acta Biomater 2012;8:836–42.
[90] Zhang X, Neoh KG, Lin CC, Kishen A. Remineralization of partially demineralized dentine substrate based on a biomimetic strategy. J Mater Sci Mater Med 2012;23: 733–42.
[91] Krifka S, Spagnuolo G, Schmalz G, Schweikl H. A review of adaptive mechanisms in cell responses towards oxidative stress caused by dental resin monomers. Biomaterials 2013;34:4555–63.
[92] de Souza Costa CA, do Nascimento AB, Teixeira HM. Response of human pulps following acid conditioning and application of a bonding agent in deep cavities. Dent Mater 2002;18:543–51.
[93] de Souza Costa CA, Teixeira HM, Lopes do Nascimento AB, Hebling J. Biocompatibility of resin-based dental materials applied as liners in deep cavities prepared in human teeth. J Biomed Mater Res B Appl Biomater 2007;81:175–84.
[94] Tay FR, Pashley DH. Aggressiveness of contemporary selfetching systems: I. Depth of penetration beyond dentin smear layers. Dent Mater 2000;17:296–308.
[95] Guo X, Peng Z, Spencer P, Wang Y. Effect of initiator on photopolymerization of acidic, aqueous dental model adhesives. J Biomed Mater Res A 2009;90:1120–7.
[96] Hashimoto M, Ito S, Tay FR, Svizero NR, Sano H, Kaga M, et al. Fluid movement across the resin–dentin interface during and after bonding. J Dent Res 2004;83:843–8.
[97] Jandt KD, Sigusch BW. Future perspectives of resin-based dental materials. Dent Mater 2009;25:1001–6.
[98] Aguilar P, Linsuwanont P. Vital pulp therapy in vital permanent teeth with cariously exposed pulp: a systematic review. J Endod 2011;37:581–7.
[99] Schroder U. Effects of calcium hydroxide-containing pulp-capping agents on pulp cell migration, proliferation, and differentiation. J Dent Res 1985;64:541–8.
[100] Torabinejad M, Watson TF, Pitt Ford TR. Sealing ability of a mineral trioxide aggregate when used as a root end filling material. J Endod 1993;19:591–5.
[101] Lee SJ, Monsef M, Torabinejad M. Sealing ability of a mineral trioxide aggregate for repair of lateral root perforations. J Endod 1993;19:541–4.
[102] de Souza Costa CA, Duarte PT, de Souza PP, Giro EM, Hebling J. Cytotoxic effects and pulpal response caused by a mineral trioxide aggregate formulation and calcium hydroxide. Am J Dent 2008;21:255–61.
[103] Bakland LK, Andreasen JO. Will mineral trioxide aggregate replace calcium hydroxide in treating pulpal and periodontal healing complications subsequent to dental trauma? A review. Dent Traumatol 2012;28:25–32.
[104] Darvell BW, Wu RC. "MTA" – an hydraulic silicate cement: review update and setting reaction. Dent Mater 2011;27:407–22.
[105] Peng W, Liu W, Zhai W, Jiang L, Li L, Chang J, et al. Effect of tricalcium silicate on the proliferation and odontogenic differentiation of human dental pulp cells. J Endod 2011;37:1240–6.
[106] Zanini M, Sautier JM, Berdal A, Simon S. Biodentine induces immortalized murine pulp cell differentiation into odontoblast-like cells and stimulates biomineralization. J Endod 2012;38:1220–6.
[107] Atmeh AR, Chong EZ, Richard G, Festy F, Watson TF. Dentin–cement interfacial interaction: calcium silicates and polyalkenoates. J Dent Res 2012;91:454–9.
[108] Tran XV, Gorin C, Willig C, Baroukh B, Pellat B, Decup F, et al. Effect of a calcium-silicate-based restorative cement on pulp repair. J Dent Res 2012;91:1166–71.
[109] Beyth N, Yudovin-Farber I, Bahir R, Domb AJ, Weiss EI. Antibacterial activity of dental composites containing quaternary ammonium polyethylenimine nanoparticles against *Streptococcus mutans*. Biomaterials 2006;27:3995–4002.
[110] Cochrane NJ, Reynolds EC. Calcium phosphopeptides – mechanisms of action and evidence for clinical efficacy. Adv Dent Res 2012;24:41–7.
[111] Nakashima S, Yoshie M, Sano H, Bahar A. Effect of a test dentifrice containing nano-sized calcium carbonate on remineralization of enamel lesions in vitro. J Oral Sci 2009;51:69–77.
[112] Tschoppe P, Zandim DL, Martus P, Kielbassa AM. Enamel and dentine remineralization by nano-hydroxyapatite toothpastes. J Dent 2011;39:430–7.
[113] Hannig M, Hannig C. Nanotechnology and its role in caries therapy. Adv Dent Res 2012;24:53–7.
[114] Cordeiro MM, Dong Z, Kaneko T, Zhang Z, Miyazawa M, Shi S, et al. Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth. J Endod 2008;34:962–9.
[115] Rosa V, Della Bona A, Cavalcanti BN, Nör JE. Tissue engineering: from research to dental clinics. Dent Mater 2012;28:341–8.
[116] Sakai VT, Zhang Z, Dong Z, Neiva KG, Machado MA, Shi S, et al. SHED differentiate into functional odontoblasts and endothelium. J Dent Res 2010;89:791–6.
[117] Zheng Y, Wang XY, Wang YM, Liu XY, Zhang CM, Hou BX, et al. Dentin regeneration using deciduous pulp stem/progenitor cells. J Dent Res 2012;91:676–82.