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Fluoride release kinetic from dental restorative materials affects Dental Pulp Stem Cells behavior

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Summary

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Abstract

Fluoride-releasing restorative dental materials can be beneficial to remineralize dentin and help prevent secondary caries [1]. However, commercialized fluoride-restorative materials (F-RMs) exhibit a non-constant rate of fluoride release depending mainly on the material composition and fluoride content [2]. Here we investigate whether different fluoride release kinetics from new dental resins could influence the behavior of human dental pulp stem cells (hDPSCs). The innovation consists in using as dental composites fillers modified hydrotalcite intercalated with fluoride ions (LDH-F). The fillers were prepared via ion exchange procedure and the LDH-F inorganic particles (0.7, 5, 10, 20 wt.%) were mixed in a commercial light-activated restorative material (RK), provided by Kerr s.r.l. (Italy) to obtain the final resins (RK-F). The physical-chemical characteristics, the release profile and the biological effect on proliferation of hDPSCs of RK-F 0.7, 5, 10 were analyzed. Since RK-F10 and a commercial fluoride-glass filler (RK-FG10) contain the same concentration of fluoride, RK-F10 was chosen to investigate the regenerative capability induced by fluoride-controlled release. To evaluate the difference between RK-F10 and RK-FG10 in inducing cellular migration and differentiation, it was isolated the human dental pulp stem cell subpopulation (STRO-1 positive cells) known for its ability to differentiate towards an odontoblast-like phenotype [3]. The release of fluoride ions was determined in physiological medium and artificial saliva medium using an ion chromatograph. The cell migration assay was performed in presence of transforming growth factor β1 (TGF-β1) and stromal cell-derived factor-1 (SDF-1) using a modified Boyden Chamber method on STRO-1^{+} cells cultured for 7 days on RK, RK-F10 and RK-FG10 materials. The expression patterns of dentin sialoprotein (dspp), dentin matrix protein 1

(dmp1), osteocalcin (ocn), and matrix extracellular phosphoglycoprotein (mepe) were assessed by quantitative RT-PCR. The incorporation of LDH-F in commercial-dental resin significantly improved the mechanical properties of the pristine resin, in particular at 37°C. Long-term exposure of STRO-1^+ cells to a continuous release of low amount of fluoride by RK-F10 increases their migratory response to TGF-β1 and SDF-1, both important promoters of pulp stem cell recruitment [4]. Moreover, the expression patterns of dspp, dmp1, ocn, and mepe indicate a complete odontoblast-like cell differentiation only when STRO-1^+ cells were cultured on RK-F10. On the contrary, RK-FG10, characterized by an initial fluoride-release burst and reduced lifetime of the delivery, did not elicit any significant effect both on STRO-1^+ cell migration and differentiation. Taken together our results demonstrated that STRO-1^+ cell migration and differentiation into odontoblast-like cells was enhanced by the slower fluoride-releasing material (RK-F10) compared to RK-FG10, which showed a more rapid fluoride release, thus making LDH-F a promising filler for evaluation in clinical trials of minimally invasive dentistry.

Riassunto

“La cinetica di rilascio del fluoro da materiali per la restaurativa dentale influenza il comportamento delle cellule staminali pulpari.”

L'odontoiatria restaurativa-conservativa si occupa di ricostruire gli elementi dentali che hanno perso parte della loro struttura in seguito a carie o eventi traumatici, utilizzando tecniche di eliminazione della carie e di restauro votate al minimo intervento ed al risparmio biologico. Infatti, mediante tale approccio nasce il concetto di “Minima Invasività” che prevede la rimozione dei tessuti colpiti dalla carie e la preservazione di quelli integri, che serviranno da solida base funzionale per effettuare un restauro del dente, efficiente, durevole ed integrato sia sotto il profilo biologico che estetico [5]. La presenza di odontoblasti nel tessuto sano consente, infatti, la rimineralizzazione fisiologica del tessuto demineralizzato, attraverso la deposizione di una matrice collagenica capace di legare ioni calcio e/o fluoro [6].

L’utilizzo di questa nuova tecnica ha contribuito allo sviluppo di materiali dentali in grado di rilasciare molecole ad azione antibatterica e/o rimineralizzante come il fluoro [2, 7]. Tuttavia, la maggior parte dei materiali oggi in commercio presenta una cinetica di rilascio di fluoro variabile, caratterizzata da un rapido rilascio iniziale seguito da un altrettanto rapido declino [8]. Studi recenti hanno dimostrato che l’esposizione iniziale ad alte concentrazioni di fluoro porta ad effetti citotossici sulle cellule della polpa dentale [9]. Pertanto, uno dei problemi ancora aperti relativi alla formulazione di materiali fluorurati è la modulazione della concentrazione degli ioni fluoruro.

Partendo da queste premesse, il progetto di tesi è stato finalizzato alla progettazione, sintesi e caratterizzazione (chimico-fisica e biologica) di materiali dentali, capaci di rilasciare fluoro con una cinetica di rilascio controllata e prolungata nel tempo. Tali concentrazioni potrebbero indurre la rigenerazione del tessuto adiacente al restauro limitando, così, le infiltrazioni batteriche ed il rischio di sviluppare una carie secondaria. Il materiale sintetizzato deve essere in grado, inoltre, di richiamare cellule staminali normalmente presenti nella polpa dentale inducendone il differenziamento in odontoblasti maturi. In particolare, le cellule che esprimono il marker di membrana STRO-1 (stromal precursor antigen-1) hanno una maggiore capacità di differenziare nei tessuti duri del dente[3].

Per la sintesi del materiale sono state utilizzate come riempitivi (fillers) compositi inorganici sintetici quali le idrotalciti, note anche come argille anioniche o idrossidi doppi lamellari (layered double hydroxide, LDH), in cui le cariche positive delle lamelle vengono bilanciate da anioni fluoro (F-) sistemati negli spazi interstrato (LDH-F). Le LDH, sintetizzate in forma nitrata, sono state caricate con sodio floruro (NaF) fino a saturazione utilizzando il metodo dello scambio anionico. Il riempitivo ottenuto è stato disperso in quantità differenti (0.7, 5, 10 e 20 % p/p) in una resina acrilica commerciale foto-polimerizzabile fornita dalla Kerr – Italia (RK). I campioni ottenuti sono stati denominati RK-Fx, dove x rappresenta la quantità di filler disperso nella resina e sono stati resi in forma di dischi di 15 mm di diametro e 1mm di spessore per poi essere valuati dal punto di vista fisico-chimico mediante l'analisi ai raggi X, l'analisi ad infrarosso in trasformata di Fourier e l'analisi del modulo elastico.

La cinetica di rilascio del fluoro, ottenuta a 37°C in un mezzo minerale con composizione simile a quello salivare, è stata misurata utilizzando uno ionometro. Le misure sono state effettuate dopo ogni ora nelle prime otto ore,

ogni giorno per la prima settimana e ogni settimana fino alla fine dell'esperimento (21 giorni) sia su RK-Fx che su una resina caricata con concentrazioni sovrapponibili di fluoro utilizzando un filler commerciale (RK-GF10). Per tutte le resine utilizzate è stata osservata una cinetica di rilascio dipendente dal tempo. In particolare, RK-GF10 presenta un rilascio rapido nelle prime ore raggiungendo concentrazioni di fluoro pari a 2.723 ± 0.163 ppm già dopo un giorno d'incubazione con piccoli incrementi nei giorni successivi. Al contrario RK-F10 mostra un basso rilascio di fluoro che gradualmente aumenta fino alla fine dell'esperimento raggiungendo concentrazioni pari 1.667 ± 0.116 ppm. La capacità del fluoro rilasciato di richiamare le DPSCs STRO-1^+ ai margini del restauro è stata valutata utilizzando un test di chemiotassi in presenza del fattore di crescita β-trasformante 1 (TGFβ-1) e del fattore derivato da cellule stromali 1α (SDF-1). I risultati dimostrano che le cellule coltivate su RK-F10 presentano una capacità di migrazione significativamente più alta rispetto a quelle coltivate su RK-FG10.

L'effetto della differente cinetica di rilascio del fluoro sul differenziamento delle cellule STRO-1^+ in senso odontoblastico è stato valutato sia mediante PCR quantitativa utilizzando markers del differenziamento sia precoci che tardivi, sia mediante colorazione con "Alizarin Red S", un colorante specifico per la matrice mineralizzata. Come markers sono state utilizzate: l'osteocalcina (OCN), la proteina della matrice (DMP1), la sialoproteina (DSP) e la fosfoproteina (DPP) della dentina e la fosfoglicoproteina della matrice extracellulare (MEPE). Dopo 28 giorni di coltura su dischi di RKF10 o RK-GF10, i risultati ottenuti dimostrano che l'espressione dei markers tardivi quali ocn, dspp, dmp1 è significativamente elevata soltanto nelle cellule coltivate su RK-F10. Sebbene l'espressione del marker precoce Mepe sia presente nelle cellule coltivate su entrambi i materiali nei primi tre giorni di coltura, soltanto nelle

cellule coltivate su RK-F10 si osserva un decremento della sua espressione nei successivi 25 giorni di coltura. Nell'insieme questi dati dimostrano che l'utilizzo di fillers in grado di rilasciare il fluoro in modo lento e prolungato nel tempo promuove, non soltanto il differenziamento di cellule staminali della polpa dentale in odontoblasti maturi, ma anche la loro migrazione verso la zona ai margini del restauro.

Outline of the thesis

Dental caries (or tooth decay) is one of the most prevalent chronic diseases of people worldwide; indeed all the individuals can be susceptible to the development of this pathology during their lifetime [10].

The caries process starts with dissolution of the tooth's mineral, called destruction/demineralization, generated by direct exposure of the tooth to the acid produced by oral bacteria (e.g., *Streptococcus Mutans*). The loss of mineral from tooth structure causes microporous areas and then a cavity develops [11].

Primary prevention consists in reducing the common risk as physical, biological, environmental, behavioural, and lifestyle-related factors such as, insufficient fluoride exposure and poor oral hygiene [10].

The treatment of caries is based on clinically intervention. Nowadays, Minimally invasive dentistry (MID) surgery consists in the selective removal of caries-infected tissue, while leaving intact the caries-affected tissue, since the caries-affected dentin can be physiologically remineralized due to the presence of viable odontoblast cells in the inner layer [6, 11, 12]. According to this concept, the common adhesive and biomimetic restorative materials have evolved in bioactive materials able to promote the remineralization of dental lesions [13].

T. Wurtza *et al*. studies highlighted the biological effect of fluoride, showing that this ion could affects odontoblast gene expression, a key for inducing tissue regeneration [14]. However, Chang and Chou described the cytotoxic effects of high fluoride concentration in the human pulp cells proliferation, mitochondrial activity, and protein synthesis [3].

Indeed, the limit of fluoridate commercial materials used for dental restoration is that they are characterized by a *burst* release of fluoride (high concentration in a short period of time) [7].

The aim of this research project is the synthesis of an innovative restorative dental material able to stimulate tissue repair by inducing migration and differentiation of dental pulp stem cells. In particular, the regeneration of the dental tissue close to restoration margins might guarantee the longevity of the treatment and preserve from secondary caries development.

1. Curriculum Vitae of Teeth: Evolution, Generation, Regeneration.

From the anatomy to the pathology and the therapy of teeth

1.1. Organogenesis and anatomy of teeth

Tooth development is the complex process by which teeth form from embryonic cells, grow, and erupt into the mouth. Teeth are generated through highly orchestrated mutual inductive interactions between two major cell types: stomodeal ectoderm and cranial, neural crest-derived ectomesenchyme cells. Morphological differences between individual teeth of a dentition arise mainly from differences in the spatiotemporal expression of several, odontogenic genes. These genes encode transcription factors that regulate the synthesis of various signaling factors. These signaling factors mediate inductive interactions between the odontogenic tissue layers and affect cell multiplication, cell death and cytodifferentiation [15]. How these inductive interactions were modified during evolution to generate the numerous anatomical features of teeth is a major interest in evolutionary biology. Indeed, the complicated, sequential, reciprocal interactions between the dental epithelium and dental ectomesenchyme that are required for tooth formation are mediated by the spatiotemporal expression of tooth-related genes (approx. 300) and the secretion of growth and transcription factors (approx. 100) that are reiteratively used in regulatory loops [16]. Epithelial cells secrete specific sets of growth factors [e.g., FGFs (FGF3, FGF4, FGF8, FGF10), BMPs (BMP2, BMP4)] and signaling molecules [SHH and WNTs (WNT3, WNT7, WNT10)] some of which regionalize the oral ectoderm (FGF8: molar=proximal=posterior domain, BMP4: incisor=distal=anterior=mesial domain) before the arrival of the cranial neural crest cells (fig.1) [17].

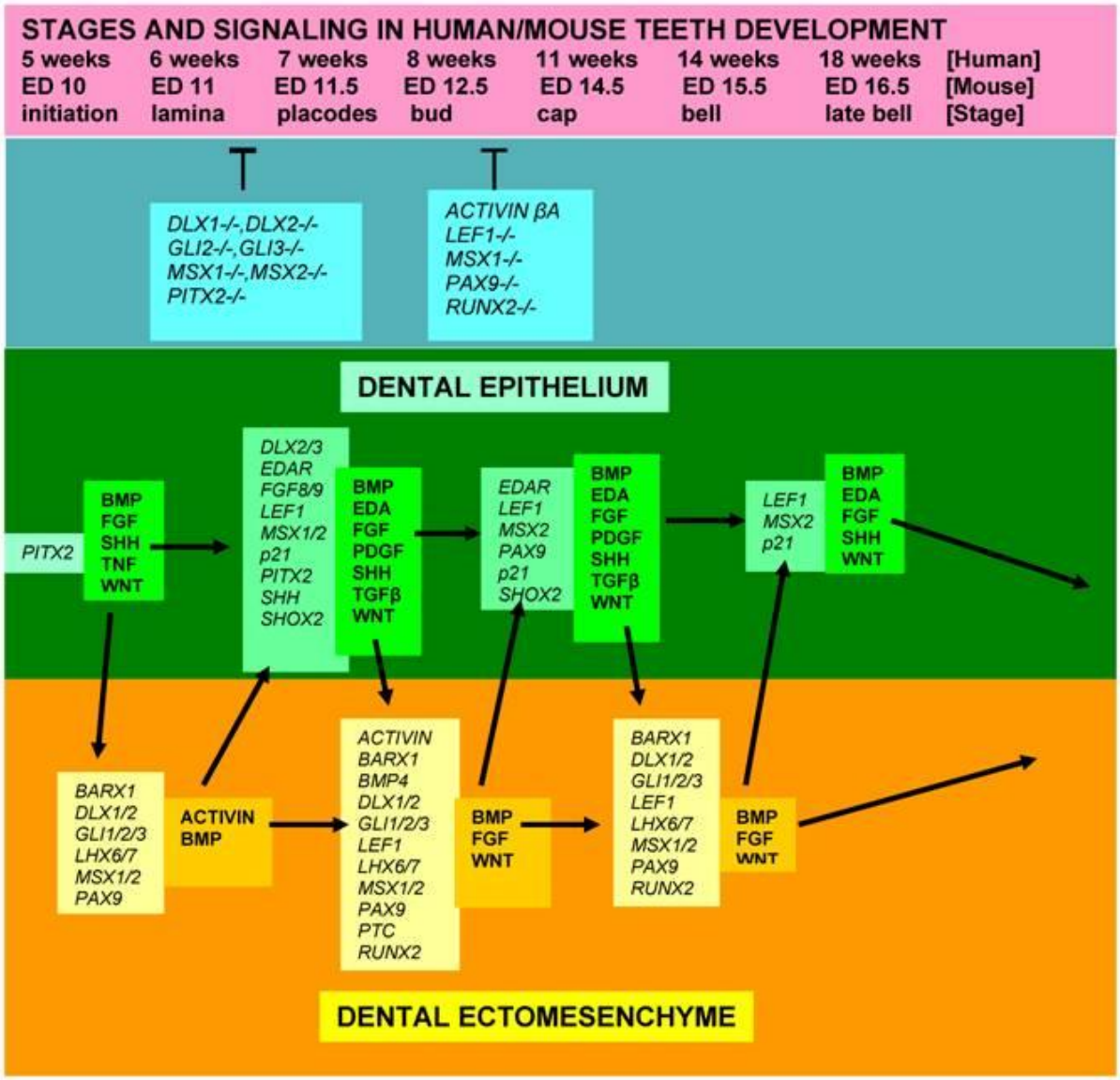

Fig.1: Stages and signalling pathways of teeth development in human and mouse. While most teeth-related genes exhibit, in general, similar expression patterns in the developing teeth in both humans and mice, several genes, including *MSX1*, *FGF8*, *PAX9*, and *SHOX2*, show some slightly different expression profiles]. [Arrows = activation; (T) = inhibition at the indicated stages; *italic* fond = genes; regular, **bold** fond = growth factors]. (Image reported from. Koussoulakou D.S.*et al*. 2009 [17])

Primary teeth start to form between the sixth and eighth weeks in utero, and permanent teeth begin to form in the twentieth week in utero. If teeth do not start to develop at or near these times, they will not develop at all. The tooth

germ is an aggregation of cells that eventually forms a tooth and is organized into three parts: the enamel organ, the dental papilla and the dental follicle [18].

The erupted tooth shows both a section in the oral cavity and in a cavity of the bone, the alveolus. A complex specialized ligament, periodontal ligament, supports teeth in the alveolus. Each tooth may be divided in an upper portion, the crown, and a bottom one, the dental root completely included in the dental alveolus (Fig. 1) [19]. The boundary zone between the crown and the root is called the neck. The bulk of the dental hard tissue is dentin, which covers the dental pulp lying at the core of the tooth. Enamel is the outer layer that surrounds the dentin in the crown area and cementum is the outer layer, covering the dentin in the root area [20].

Teeth are composed of four tissues: enamel, dentin, and cementum that are the hard mineralized tissues, and the pulp that is the soft one (fig.1) [21].

- **Enamel** is the hardest tissue in the body, thus making the tooth able to withstand a great amount of stress and temperature change. This translucent tissue is made up of 96 wt% inorganic materials; the main part is composed by carbonated hydroxyapatite crystals, while sodium, magnesium, chlorine, carbonate, potassium and fluoride represent trace elements. Indeed, enamel formation process, the *Amelogenesis*, consists in hydroxyapatite crystals precipitation and growth initiated by the secretory activity of the ameloblasts (enamel forming cells) into the extracellular space adjacent to the dentino-enamel junction [20]. However, enamel shows an acellular and avascular structure without the capability to regenerate or repair itself, but it can remineralize. This means that areas experiencing early demineralization (loss of minerals) are able to regain minerals and stop the caries process. Demineralization

and remineralization can occur without loss of tooth structure when proper nutrition and oral care are respected [19].

- **Dentin** is a hydrated tissue, softer than enamel but harder than bone. It consists of approximately 50 vol% of carbonated hydroxyapatite (Hap) minerals, 30 vol% of collagen and noncollagenous molecules and 20 % of water [20]. The 90 wt% of the organic phase in dentin is almost composed of collagen type I. A series of synchronized biological events lead to dentin formation. This process, known as *Dentinogenesis*, is induced by odontoblasts that differentiate from ectomesenchymal cells of the dental papilla. The dental papilla primarily generates the formation of dentin until it is finally surrounded by secreted dentin, thus forming the dental pulp later [20]. There are three types of dentin: primary, secondary and tertiary. Primary dentin forms when a tooth erupts. Unlike enamel, dentin can furtherly grow forming secondary dentin. This secondary dentin will continue to grow throughout the life of the tooth. Tertiary dentine, also known as reparative dentin, forms as a response to irritation and trauma such as erosion and dental caries [19].
- **Cementum** is a mineralized avascular connective tissue, similar in composition to bone; indeed, it is composed of 65 wt% of Hap minerals, 23 wt% of organic matrix and 23 %wt of water. The organic substance consists in proteoglycans and glycoproteins for the amorphous part, and in collagen fibers for the structured one. Cement is secreted during *Cementogenesis* by cementoblasts, which are cells that share a similar morphology with odontoblasts. This tissue cannot be repaired [20]
- **Dental pulp** is a mucous connective tissue contained in the pulp chamber limited by dentin. Dental pulp and dentin have got the same

embryonic origin. Due to its similarity to mesenchyme, the pulp is mainly composed by amorphous gelatinous ground substance, rich in glycoproteins, proteoglycans and glycosaminoglycans (mostly hyaluronic acid) and few fibroblasts. It is located within the cavity called pulp that is surrounded by odontoblasts and dentin. It continues in the root forming the root pulp. The dental pulp is the best cell source for tissue engineering, since it is a rich reservoir of stem cells residing in various areas (mainly in root) and having numerous plasticity characteristics [22, 23].

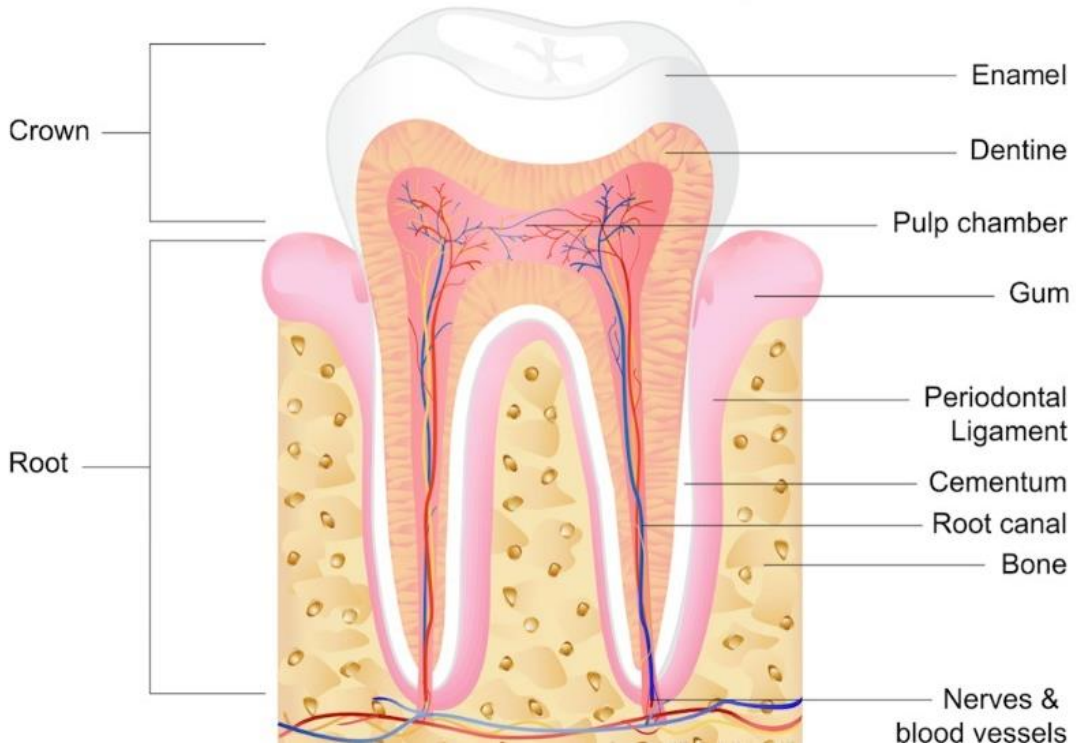

Fig.1: Divisions and tissues of the tooth

1.2. Teeth pathologies.

Dental Pathology refers to any dental condition that is either congenital or acquired. Congenital pathologies include tooth abnormalities as Anodontia, a rare genetic disorder in which the patient shows absence of all primary or permanent teeth. Acquired conditions are more frequent than the congenital pathologies and they are relatively simpler to be restored. In particular, Dental Caries (or tooth decay) and Dental Abscess, both caused by bacteria, are the most common diseases [24, 25].

Dental caries is a process of destruction of the tooth hard tissues caused by bacteria. Teeth surface is coated by an acquired pellicle, which consists of lipids and proteins, including salivary glycoprotein. The pellicle is recognized by primary colonizing bacteria that express receptors for the glycoprotein as *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus gordonii* and *Streptococcus sanguis*. These bacteria form an initial biofilm of few layers that gradually grow with the appearance of later colonisers that use receptors and adhesins to create the final biofilm or dental plaque. Dental plaque consists of mostly gram-positive cocci bacteria, followed by some gram-positive rods and fillaments and a very small amount of gram-negative cocci. The gram-positive species as *Streptococci* (in particular *S. mutans*) and *Lactobacillus casei* and the gram-negative *Actinomyces* are the most involved bacteria with caries development [10, 26]. The initial biofilm is almost always present on the tooth surface as it forms immediately after cleaning (fig.2).

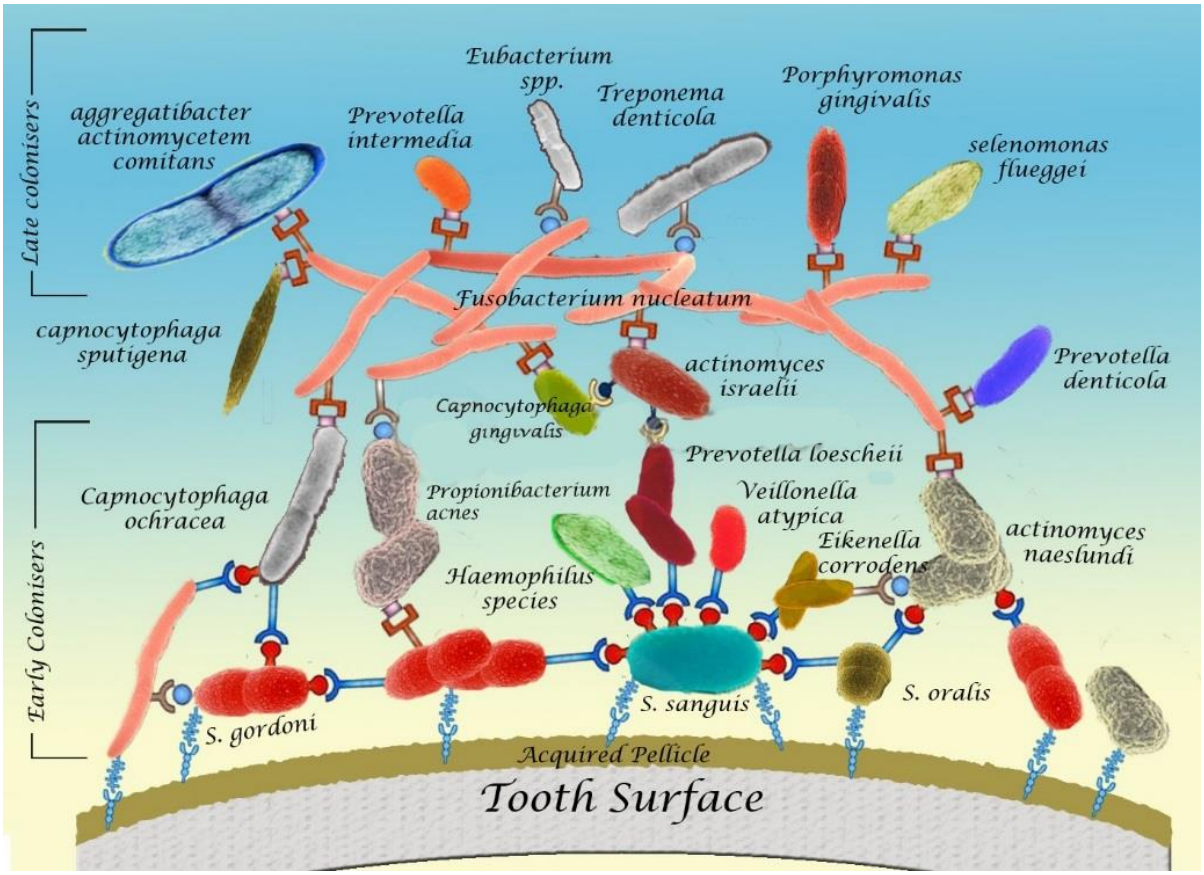

Fig.2: A model of oral biofilm formation (reproduced from http://periobasics.com/).

Indeed, in tooth decay, fermentable carbohydrates derived from diet stimulate the metabolic activity of the biofilm bacteria and generate pH variation in its fluid. Considering that, when the pH of biofilm fluid (or in general into saliva) is below a critical value of 5.5, tooth enamel demineralized. However, if it is above the critical value, the fluid is considered supersaturated and favors crystallization of the mineral and the remineralization. Persistent variations can create an imbalance between tooth mineral and biofilm fluid, resulting in a mineral loss and lesion formation (fig.3) [27].

Erosion, abrasion and wear are also characterized by the phenomenon of progressive demineralization, but differently from caries lesions are not caused by dental plaque[28-30].

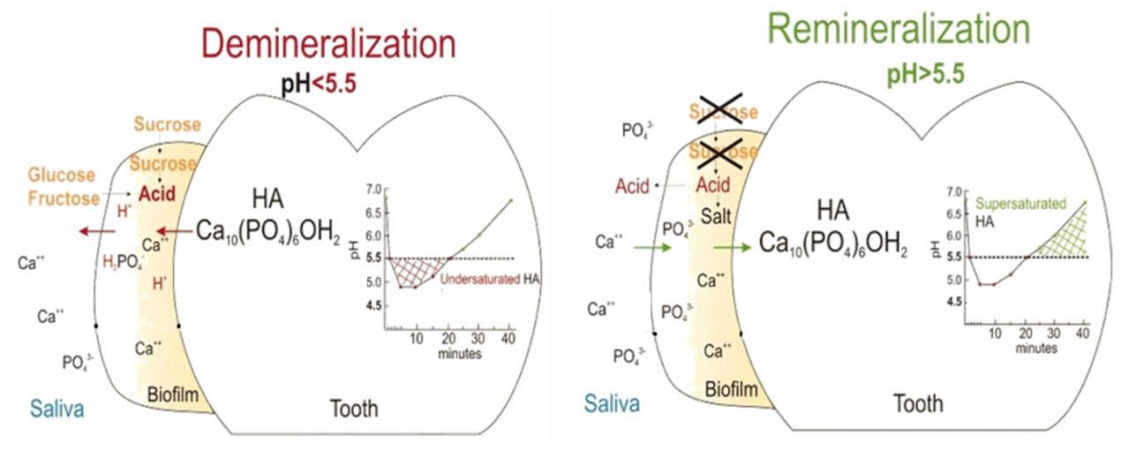

Fig. 3: Enamel remineralization in dental biofilm. Sugars (sucrose, glucose, fructose) are converted to acids in the biofilm. When the pH decreases to below 5.5, undersaturation with respect to hydroxyapatite (HA) is reached in the biofilm fluid, resulting in mineral dissolution. After exposure to sugars has ceased, acids in the biofilm are cleared by saliva and converted to salts. As a result, the pH increases, and at pH 5.5 or above, the biofilm fluid is supersaturated with respect to HA. If these two phases are not in equilibrium, caries develop. (Image adapted from Cury and Tenuta, 2008 [31]).

Dental caries that occur for the first time in a healthy tooth is defined primary caries, while secondary caries refers to the developing of a tooth decay adjacent to an existing restoration site (fig.4) [32].

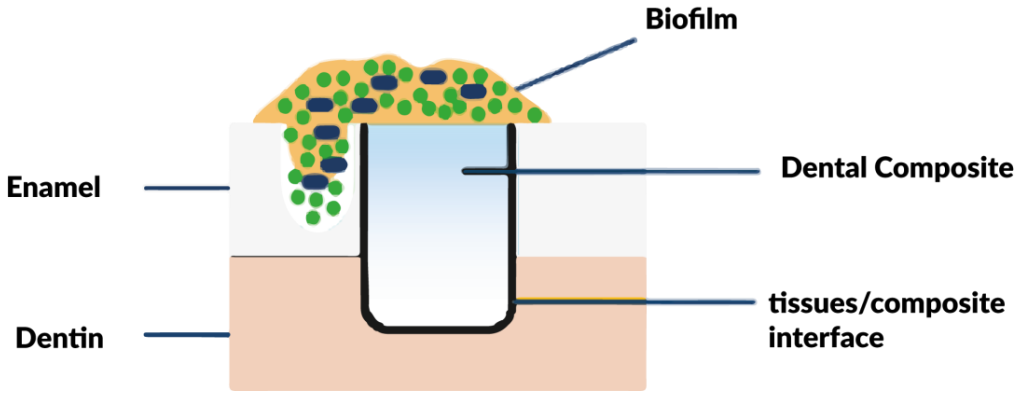

Fig.4: The diagram of secondary caries development by biofilm in the tissues next to the restorative material.

(Image adapted from Kuper 2015 http://hdl.handle.net/2066/139138 [32])

Secondary caries can develop in two manners: as lesions on the tooth surface close to restoration margins (similarly to primary caries), or as wall lesions if there are gaps in the tooth/restoration interface (fig.5) [33].

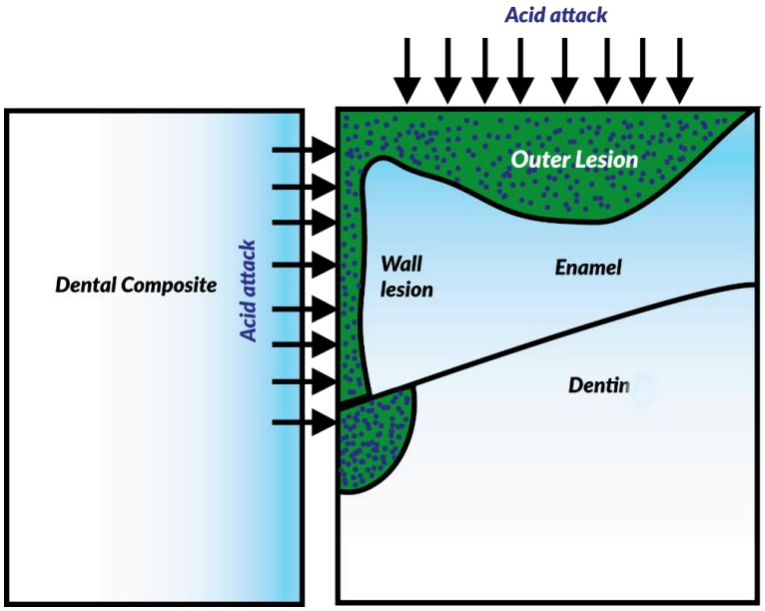

Fig. 5: Schematic representation of injuries related to the secondary caries lesions and external wall

(Image adapted from Kuper 2015 http://hdl.handle.net/2066/139138 [32])

The first interpretation of the relationship between the gaps and the lesion wall was the theory of hydrodynamic flow. This assumption recognized the not bonded tooth-restoration interface as sensitive site, subjected to opening and closing forces that created a hydrodynamic flow. The dissolution products derived from a started demineralization moved with this flow and did not create a steric hindrance inside the gap. This situation allowed a new acid attack and secondary caries progressed faster [34]. Two more recent theories are hypothized to explain this relationship gaps/lesion wall. The first one is the "theory of micro-infiltration" that indicates, as cause of the demineralization and the consequent lesion wall, the passage of small amounts of fluid into the gaps of the tooth/restoration interface. Bacteria carried with the fluid are able to reach the sensible site and cause pathogenicity. The second hypothesis, the "theory of macro-infiltration", assumes that bacteria micro-infiltration is not sufficient for the lesion development. Differently, it suggests the necessity of a stable settlement of the biofilm in the gap to have considerable demineralization. Moreover, gap's dimensions are expected to be from 225 micrometers to 400 micrometers to allow bacteria settlement [35].

1.3. Dental caries treatment

The classical therapeutic approaches include extrinsic dental interventions, such as tooth filling, tooth extraction and implantation of an inert, artificial (metal, ceramic) substitute. Those interventions are not always free of unpleasant and/or adverse side-effects [36]. Therefore, an ambitious dream of numerous dentists is to be able to substitute the artificial inert material with a bioactive one that is able to stimulate a genuine replica of the damaged tooth part. In this respect, regenerative medicine, which seeks ways to imitate natural physiological mechanisms of organ initiation and morphogenesis, could be of help.

1.3.1. DENTAL CARIES PREVENTION

Dental caries prevention consists in a correct oral hygiene, in reducing sugar intake and in using fluoride sources (toothpaste, mouthrinse, water and professional topical fluoride application). Fluorides are very effective in preventing dental caries, primarily by inhibiting mineral loss from the tooth. However, the application of all these measures do not always inhbit tooth decay development [37]. Even dental caries prevalence has declined during the past few decades, it remains still one of the major oral chronic human diseases in the most industrialized countries [38].

According to modern dentistry, dental caries treatment provides to manage non-cavitated caries lesions non-invasively to promote the remineralization of the affected tissues. Indeed, dental materials are continually improved not only for their aesthetic property, but especially for their biological effect and longevity [39].

1.3.2. MINIMAL INTERVENTION DENTISTRY

Restorative Dentistry deals with the diagnosis, prevention and treatment of congenital and acquired lesions of the teeth structure through restorative operation. On this field, recent progresses in biomaterials technology lead to a new branch of this discipline: the Conservative Dentistry. This subject is aimed to preserve natural tissues of tooth introducing a new surgical approach called the "Minimal Intervention Dentistry" (MID) in which the invasive treatments of dental structures are strongly limited. For example, regarding caries medical care, G.V. Black's theories of the last century required the complete removal of all areas of demineralized tooth structures and their subsequent reconstruction through inert restoration [40]. MID approach, instead, consists in the selective removal of infected tissues and in the preservation of healthy cells structures (caries-affected dentin) that will serve as solid support for a functional, efficient, durable and integrated restoration. Indeed, caries-affected dentin can remineralize due to the presence of viable odontoblasts in the inner layer and of the collagen network still capable of binding calcium and fluoride ions [6, 11, 12]. Such approach decreases the risk of more complex interventions of conventional prostheses for many years [5, 41, 42]. In particular, MID caries treatment is carried out removing only the dentine affected by caries at external level (dentin decomposed) and preserving the one at inner levels (demineralized dentin). Such tissue is able to remineralize using particular biomaterials [43-45].

1.3.3. THE MATERIALS FOR RESTORATIVE TREATMENT

Dental tissues, damaged by trauma or bacterial infections can be restored by the use of biocompatible synthetic materials.

Acrylic resins have been chosen as successful materials in Dentistry for their excellent biocompatibility, aesthetical properties and the easy handling. In 1949 Hagger developed the first restoring polymeric system via chemical catalysis, binding acrylic resin to dentin. This followed by a wide range of studies on dental polymers paving the way to the commercialization of poly(methyl methacrylate) (PMMA) based products [46]. However, acrylic polymers are susceptible to wear and shrinkage due to the uncomplete conversion of the monomers during polymerization reaction.[47] These phenomena lead to poor marginal seal and fractures [48].

In attempt to improve the chemical-physical proprieties of acrylic resins, in 1962 the dental restorative composites have been developed [48]. Restoration composites are made of synthetic monomers (e.g. dimethacrylate), reinforcing fillers (e.g. radiopaque glass, quartz or silica), chemical agents to promote polymerization reaction and silane as coupling agent.[49] The polymerization methods, the kind of acrylate used and the filler content strongly influence the resins features (fig. 6). Indeed, Moraes *et al* reported the photocuring treatment reduces resin shrinkage [50].

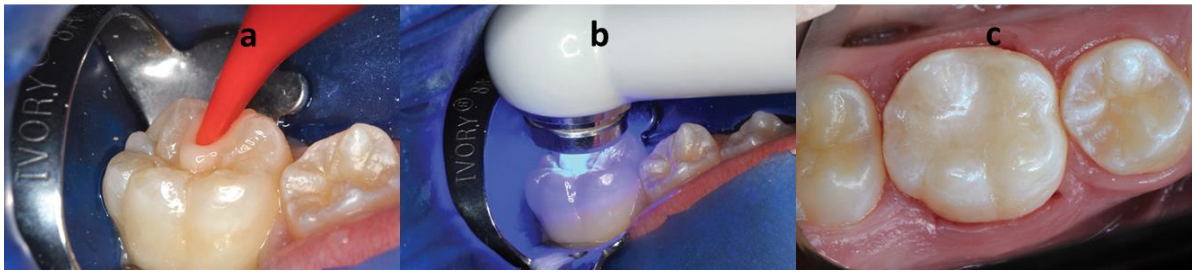

Fig.6: Restoration phases: a) material injection, b) photo-polymerization, c) resulted restoration. (Image adapted from Croll 2015 [51])

Moreover, triethylene glycol dimethacrylate (TEGDMA) or urethane dimethacrylate (UDMA) were blended with bisphenol-glycidyl methacrylate (bis-GMA) to reduce its viscosity, being the most commercialized acrylates in restorative [52].

In addition, the use of filler in dental restoration resins improved their hardness, strength, radiopacity, and workability [53]. Moreover, fillers induce a decrease in polymerization shrinkage and water sorption [54, 55].

Although the treatments and the commercially used monomers have remained largely unchanged, the most significant changes in dental resins have been in the type, size, and distribution of the fillers.

The first fillers used, representing the basis for many of the other types of fillers, were silicon dioxide, termed silica, or quartz when in its crystalline state (Table 1) [56, 57]. In addition, many other oxides have been studied including aluminium oxide (Al_2O_3), titanium dioxide (TiO_2), zinc oxide (ZnO), and zirconium oxide (ZrO_2). Nevertheless, only few of these materials have been commercialized (Table 2) [58].

Filler Type	Examples	Chemical composition
Oxides	Silica, alumina, titania, zirconia	M_xO_y
Alkaline silicate glass	Barium glass, strontium glass	$M_xO_ySiO_2$
Biomimetic filler	Hydroxyapatite	$Ca_5(PO_4)_3OH$
Organic-inorganic hybrid	ORMOCERs	SiO_2-polymer

M= metal or metalloid, x= 1-2, y= 2-3.

Table 1: filler types and their elemental compositions. (Reproduced from Habib 2016 [58])

Type of filler*	Example products using filler**
Silica	Voco Admira, Bisco micronew, Ivoclar Vivadent Artemis
Alkaline glass	Ivoclar Vivadent Artemis, Kuraray Noritake Dental Clearfil Majesty
Other glasses	Voco Admira, Shofu Beautifil II, 3 M ESPE Filtek
Prepolymerizated filler	Kuraray Noritake Dental Clearfil Majesty, Kerr Premise

Table 2: Examples of commercial filler product. * Hydroxyapatite not included due to the lack of literature on its use in commercial composite. ** Not an exhaustive list, and only representative examples are shown. (Reproduced from Habib 2016 [58]).

However, silica and metal oxide-based restorative materials were not immune from both marginal degradation during time and the consequent gap formation between the tissue/material interface [59]. This gap allowed bacteria infiltration and secondary caries development, causing restoration failure [60]. For these reasons, new dental materials have been developed in

order to release bioactive molecules having antibacterial and remineralization properties [61].

Fluoride (F^-) represent one of most used molecule since its effects on oral bacteria and plaque are well documented [62].

Indeed, F^- is able to increase the resistance to the acid environment, originated from bacterial infections, through the conversion of hydroxyapatite into fluoroapatite [63, 64]. The F^- binding to calcium hydroxyapatite (HA) conducts to enamel mineralization: penetrating into the more superficial layers of the enamel, F^- allows the anchorage between two molecules of HA, creating a more resistant structure [65]. The mechanisms of fluoride interference with bacterial metabolism and dental plaque acidogenicity include the inhibition of the glycolytic enzyme enolase and the proton-extruding ATPase as well as the bacterial colonization and competition [62]. Indeed, ATPases are proton traslocating enzymes involved in the acid-base mechanism of dental plaque bacteria. At acid pH, bacteria initiate an acid tolerance response (ATR) and thereby adapt to an acidic environment. The ATR is a phenotypic change of the bacteria and involves changes in protein expression, increased ATPase activity and shifts to lower pH optimum for glucose transport and glycolysis [66]. The presence of this acid - tolerant microflora leads to a condition of acidity in plaque that persists over time, causing enamel demineralization and caries development. Furthermore, other intracellular or plaque associated enzymes, such as acid phosphatase, pyrophosphatase, peroxidase and catalase, may be affected by fluoride ions [62].

The widespread modern fluoridated restorative composites are Glass Ionomer Cements (GICs). GICs (also referred to as polyalkanoate cements or Aluminosilicate-polyacrylic acid cements) were introduced in dentistry in 1969

by Wilson and Kent (referenza). They are composed by glassy powder based on acid-soluble calcium fluoroaluminosilicate and polyacrylic acids with copolymers in liquid form. The cement is obtained through the acid-base reaction between the glassy powder and the liquid that leads to the destruction of the glass network and to the release of ions. The released cations are chelated in the carboxylate polymer producing cross-links in the polymer network and forming a hard polysalt-matrix [67]. The bioactivity of GICs is related to their capability of linking and stabilizing teeth calcium-deficient carbonated hydroxyapatite by ion exchange. The carboxylic groups of GICs replace the phosphate ions of the teeth hydroxyapatite surface to establish ionic bonds with calcium ions derived from the partially dissolved crystals [1]. Biocompatibility, on the other hand, is achieved through the interaction of the polymerized polyacrylic acid with tooth calcium that prevents the movement of the material inside the dentinal tubules. Furthermore, GICs interfere with subgingival biofilm formation, decreasing the irritation of the periodontal tissues [68]. To improve the physical properties, such as flexural strength, reinforced glass ionomer cements were developed. Examples of reinforcing agents are fiber, metals, resins and modified amino-acids [67, 69].

Commercialized GICs (e.g. Fuji IX GP, Ketac N100, Dyract Extra and Wave) designed to release fluoride are listed in figure 7 [70].

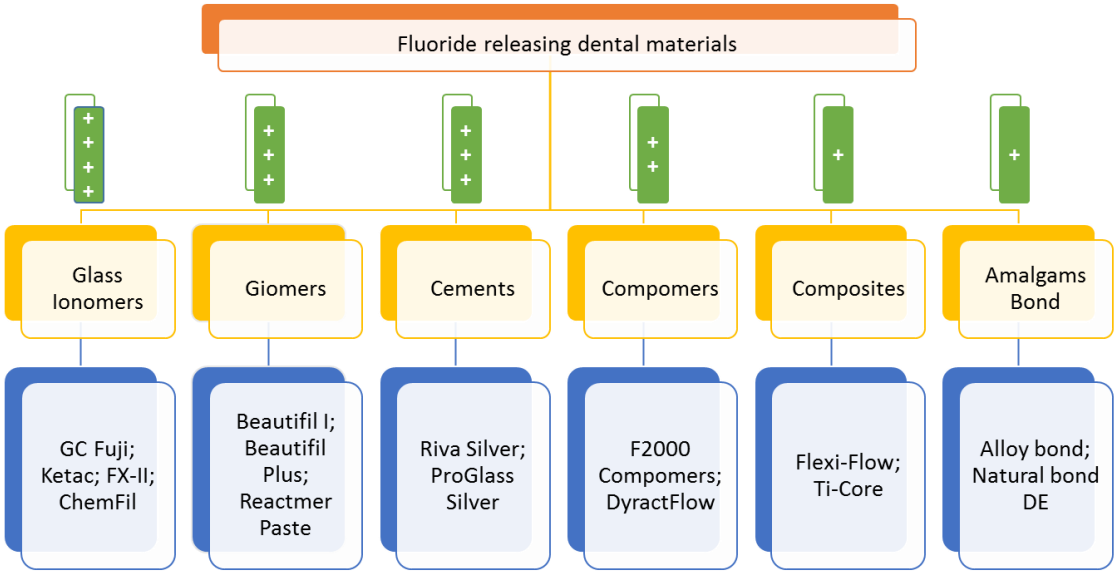

Fig.7: Fluoride releasing dental materials and their quantitative ability (+) to release fluoride in oral cavity. (Reproduced from Zafar, 2015 [71])

Hybrid materials combining the technologies of glass ionomers and composite resins were subsequently developed to help overcome the problems of conventional glass ionomer cements (GICs) and maintain their clinical advantages. Examples of these hybrid materials include resin-modified glass ionomer cements (RMGICs) and polyacid-modified composite resins (compomers). These materials have different setting mechanisms. In particular, RMGICs set by an acid-base reaction and free radical polymerization mechanisms, while compomers set by free radical polymerization only with a limited acid-base reaction occurring later as the material absorbs water from the oral environment [72]. Recently, a new category of RMGICs has been introduced for restoration of primary teeth and small cavities in permanent

teeth. The major innovation of these materials involves the incorporation of nano-technology, which allows a highly packed filler composition (~69%), of which approximately two-thirds are nano-fillers. Due to these alterations in composition some authors support that these materials belong to a new category of hybrid materials called nano-ionomers [73].

Another category of hybrid materials, which was recently introduced to the dental profession, is known as Giomers. The Giomers are constituted by a glass core coated with 3 semi-permeable layers that protect the durability and aesthetics of the glass, while allowing ions to travel freely between the glass core and the oral environment [74]. Differently from GICs, Giomers can be recharged with a new fluoride concentration. Fluoride giomers sources are toothpaste, topic gels, mouth rinse, and even fluoridated water [75].

Most *in vitro* studies have found evidence for inhibition of enamel demineralization surrounding restorations by fluoride-releasing restoratives, although they were not able to eliminate the enamel lesions. Currently, relatively few *in vivo* and *in situ* studies investigated the demineralization behavior of enamel adjacent to fluoride-releasing restorative materials. The results of these studies are not consistent, thus the clinical relevance of fluoride-releasing restoratives is still debatable [62]. Some *in vivo* studies found association of fluoride release from restorative materials with inhibition of secondary caries formation around restorations [76], but some others did not find any relation [77].

In conclusion, the caries protective effect of fluoride-releasing materials may be related to the material's ability to release adequate amounts of fluoride ions for sustained periods of time and during acidic attack. However, the fluoride releasing ability of the materials so far used is limited in time, while

the degree of protection is highest only in the closest vicinity of the restorations.

2. The evolution in the treatment of dental caries.

Is tooth regeneration a prospective clinical reality or a fantasy?

In the past, it was believed that the tooth had got limited capability to self-repair and to regenerate [19], but recent studies highlight the presence of stem cells in permanent, primary an in wisdom teeth. Dental stem cells are easy, convenient, and affordable to collect. Since these cells were used to regenerate damaged tissue in medical therapy successfully, it is possible that the dentist in future might use stem cell to regenerate lost or damaged dental structures [78].

2.1. Dental Mesenchymal Stem Cells.

In Dentistry, oral Mesenchymal Stem Cells (MSCs) are more used than Epithelial Stem Cells (ESCs) because they are able to generate a wide range of tissues [79]. The mesenchymal stem cells are non hematopoietic, multipotent cells that can proliferate and differentiate into a range of cell types comprising various tissues. They were identified in the bone marrow for the first time. These cells demonstrated specific properties such as: fibroblast-like morphology, ability to adhere on plastic tissue-culture surfaces, and osteogenic potency [80].

From the dental pulp is possible to obtain the so called Dental Pulp Stem Cells (DPSCs). DPSCs are mesenchymal cells, consequently they are responsible of the formation of different tissues of mesenchymal origin, as dentin. Nevertheless they can also evolve in melanocytes and functional neurons [81]. Other mesenchymal cells are isolated from the pulp of exfoliated deciduous teeth (SHEDs) [5]. Further extraction sites are the Apical Papilla [82], (the precursor tissue of the dental pulp), and the pulp of supernumerary teeth, which are generally discarded. It has been demonstrated that Stem Cells from

Apical Papilla (SCAPs) can differentiate into osteoblasts and odontoblasts *in vivo* [83]. Osteoblasts are the bone-forming cell [84], whereas odontoblasts secrete pre-dentin/dentin components [85].

Periodontal ligament and dental follicle are other sources of mesenchymal stem cells. However, periodontal ligament stem cells (PDLSCs) [86], and dental follicle progenitor cells (DFPCs [87] are used to regenerate periodontal ligament and cement (fig.8) [80].

Usually, the tool of regenerative dentistry is the use of DPSCs both for their substantial presence in dental pulp tissue and for the non-invasiveness of the isolation methods) [81]. The DPSCs can be isolated by explant method from tissue fragments or by enzymatic digestion of dental pulp by collagenase/dispase [81]. It was shown that the human DPSCs (hDPSCs) differentiate in a synergistic way into osteoblasts and endothelial cells [88]. Moreover, they are able to differentiate into odontoblast-like cells, characterized by the presence of polarized cell bodies and by the accumulation of mineralized nodules [88]. The different markers of mesenchymal stem cells (e.g. STRO-1, c-kit and CD34) are used to select subcategories of DPSCs that show a different biological behavior. Indeed, the cell surface protein, STRO-1 (stromal precursor antigen-1), is particularly relevant in Dentistry, because it identifies the cells (DPSC-STRO-1^{+}) that preferentially differentiate in odontoblasts [89]. Although low levels of expression of this antigen are found also in hematopoietic cells, nowadays, STRO-1^{+} is a useful marker for dental studies.

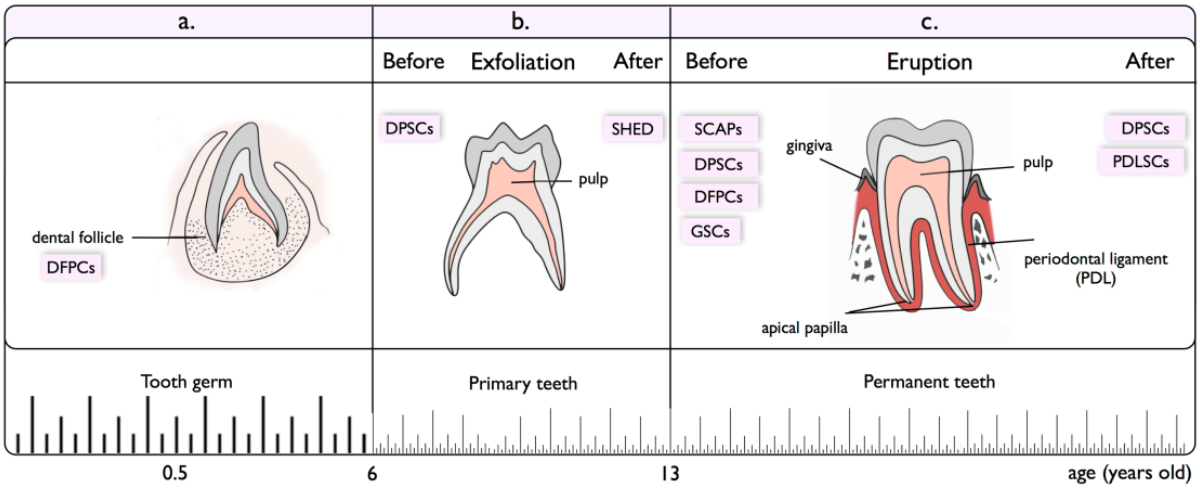

Fig.8: Overall view of dental-related stem cells based on different anatomical locations and stages during the human lifetime in. a) tooth germ, b) primary teeth, c) permanent teeth. (Reproduced from Karamzadeh and Eslaminejad, 2013 [90])

2.2. Odontoblasts.

Odontoblasts derive from ectomesenchymal cells. They exhibit a tall columnar shape and establish a continuous single layer with an epithelioid appearance. During dental morphogenesis, biochemical signals from epithelium promote the differentiation of DPSCs in pre-odontoblasts [91] and form the pre- dentin. Successively, pre-odontoblasts differentiate into odontoblasts, which start to secrete the extracellular matrix that gradually mineralizes and becomes primary dentin [92]. The odontoblasts are also responsible for secondary and tertiary dentin secretion during dentinogenesis. Changes in its secretory activity reflect differential transcriptional control [93]. After dentinogenesis, they are aligned along the periphery of the dental pulp and deposit new layers of dentine throughout life, guaranteeing the maintenance of the tooth integrity (fig.9). In addition, odontoblast-like cells, as pulp fibroblasts, may form a layer of reactionary dentine after some tissue injuries [94] that show an

atubular structure. Instead, the primary dentine exhibits a tubular organization through which the odontoblast maintains communication with its extracellular matrix environment and which is also implicated in the transmission of sensation through the tissue. The loss of this tubular structure affects tissue function [95].

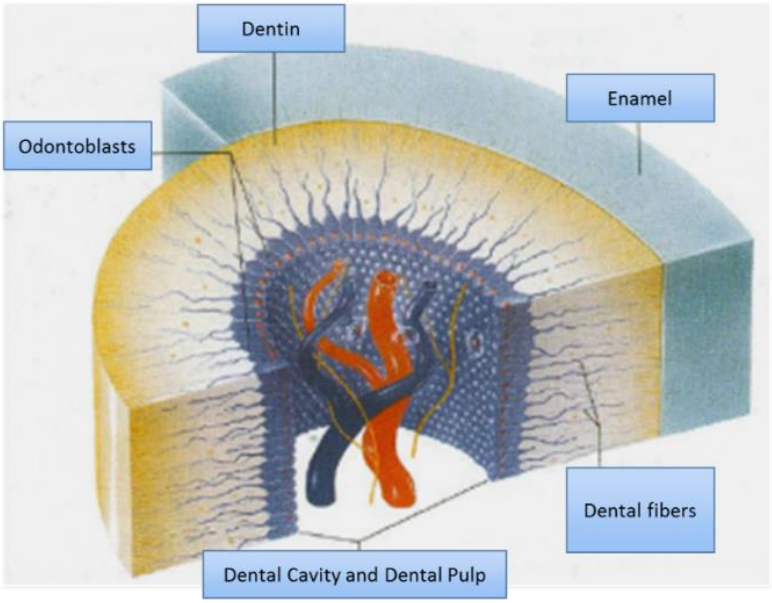

Fig. 9: Peripheral layer of odontoblasts in dental pulp

Odontoblastic differentiation is manifested by synthesis of the extracellular matrix proteins of the dentine, which includes both the collagen proteins and non-collagenous ones. The DSPP (dentin of sialo-phosphoprotein) and the DMP-1 (dentin matrix protein 1) are the most relevant marker genes of the differentation. These genes encode, respectively, for the namesake non-collagenous proteins [96]. The DSPP protein is enzymatically cleaved into DPP

(dentin phosphoprotein) and DSP (dentin sialoprotein) [97]. The two proteins are alternated during mineralization: DSP acts in the early stages, while DPP is involved in the maturation phase of the process [98].

DMP-1 is a bone and tooth specific phosphoprotein, initially identified in the mineralized dentin matrix, but it is also expressed in non-mineralized tissues. Due to its acid characteristics and its capability to bind calcium ions, DMP-1 plays a fundamental role in the hydroxyapatite nucleation within the collagen matrix of the bone and the tooth, during mineralization [99]. Furthermore, the role of DMP-1 in the odontoblastic differentiation has been widely described in the literature [100]. Indeed, the DMP-1 binding to TCF11, a leucine zipper transcription factor, allows the formation of a complex that induces reversible suppression of the expression of DSPP in undifferentiated odontoblasts [101].

Odontoblastic differentiation may be followed also evaluating the expression of other markers molecules, such as MEPE (extracellular matrix phosphoprotein) and OCN (osteocalcin). MEPE, also called osteoblast/osteocyte factor 45 (OF-45), is expressed in hard tissue, where it conducts a regulation activity on mineralization state of the extracellular matrix, acting in the early stages of differentiation. In fact, during the progress of odontoblastic maturation, its expression is down-regulated [97]. Instead, OCN (or GLA protein), a protein expressed both in bone and in tooth, acting in the late stages of the extracellular matrix mineralization, is detected in mature enamel and not in pre-dentin. OCN is involved in the inhibition of mineralization. Indeed, OCN binds calcium ion through GLA (γ-carboxyglutamic) residues, making Ca^{2+}unavailable to bind phosphate ion thus, preventing the growth of hydroxyapatite crystals [102].

Odontoblasts seem to play a role as sensor cells due to channels involved in mechanotransduction or nociception that can perceive external thermal variations or biomechanical forces. Therefore, odontoblasts maintain a narrow relationship with the living part of the tooth and can feel bacteria infiltration during caries development. Bacterial molecules are recognized by Toll-like receptors (TLRs), which are expressed in healthy human dental pulp cells. In particular, odontoblasts perceive Gram-negative bacteria involved in tooth decay using TLR-4 [103]. Moreover, *in vivo* studies show that there is an accumulation of immature dendritic cell (DC) in the odontoblast layer in response to cariogenic bacteria invasion. This means that odontoblasts initiate an innate immune response during the early stage of caries by secreting chemokines that recruit immature dendritic cells (fig 10) [104, 105]. Chemokines constitute the largest family of cytokines and have a critical role in all immune cell migration during inflammation or disease [106].

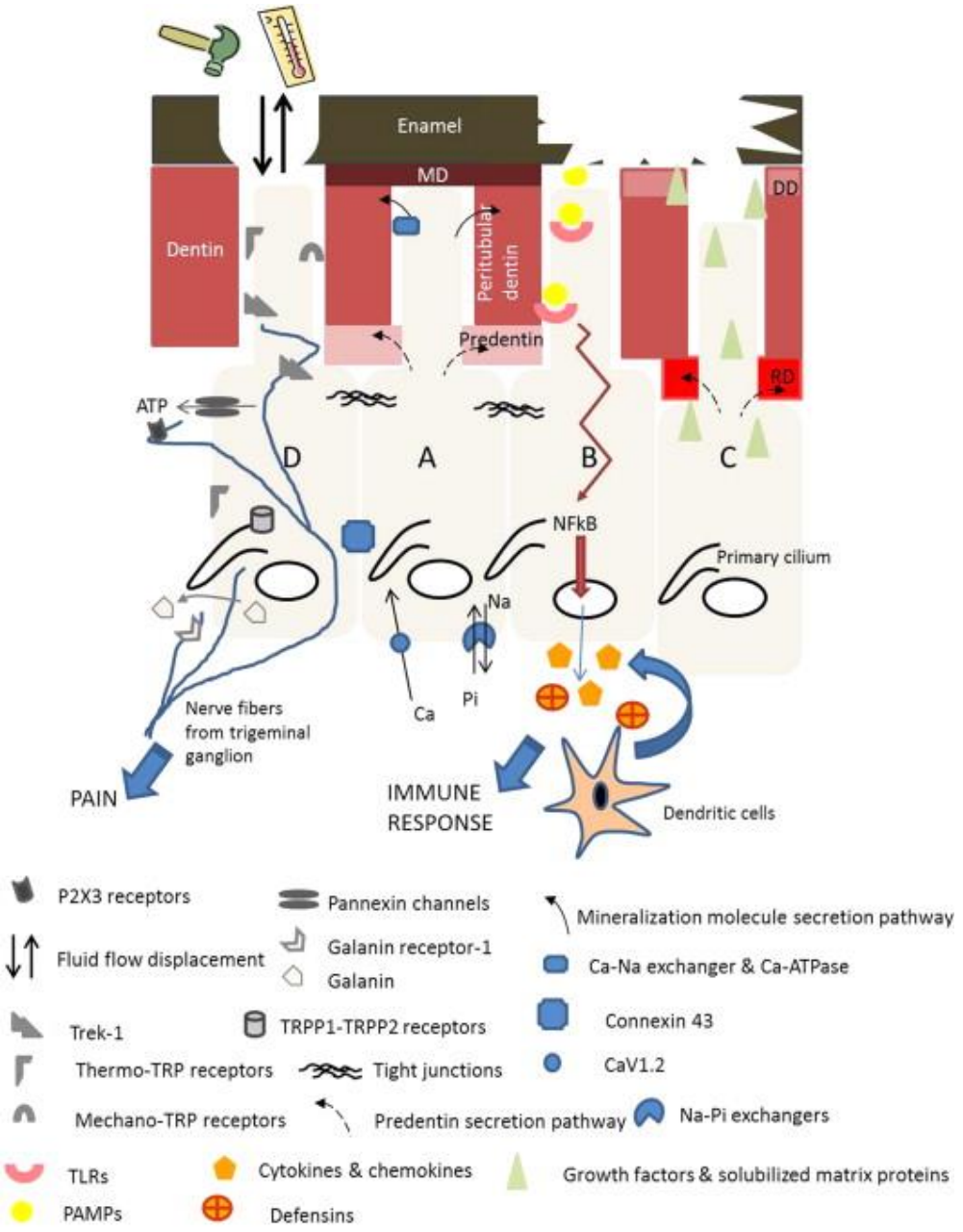

Fig.10: Schematic drawing of odontoblast activities. (A) Secretion and mineralization of the predentin–dentin matrix. (B) Initiation of the innate immune response (C) Production of reactionary dentin. (D) Activation of odontoblasts through mechanosensitive and thermosensitive channels and consequent secretion of signaling molecules. MD, mantledentin. RD, reactionary dentin. DD, demineralizeddentin. (Reproduced from Bleicher, 2014 [103])

Cellular migration induced by chemiotactic molecules represents a prerequisite for tissue damaged regeneration, inducing the recruitment of dental cells. Chemotactic molecules are not exclusively cytokines, but also growth factors and they are often interchangeably used since they share similar actions [107]. For example, T. Suzuki et al. demonstrated that dental pulp stem/progenitor cells can be induced to migrate and subsequently differentiated by three chemotactic molecules: stromal-derived factor-1α (SDF1), basic fibroblast growth factor (bFGF) or bone morphogenetic protein-7 (BMP7). In particular, the effect of SDF1 and bFGF on DPSCs migration was more evident [4]. Other studies are focused on the Transforming Growth Factor β-1 (TGFβ-1) as chemotactic molecule since it is well known that TGFβ1 is involved in a wide range of cellular activities, such as migration, proliferation, differentiation, extracellular matrix synthesis, etc. [107].

2.3. Regenerative dental materials

The vitality and dentine repair capacity of the pulp is dependent on odontoblast survival. Variations in the extent of odontoblast injury caused during surgery may be the major underlying reason for the success or failure of restorative treatments [108]. Although new dental materials show better chemical-physical and biological performance than the materials used in the past, restoration stability remains an open question [109].

This suggests that complications associated with marginal fracture and bacterial microleakage may still persist [110].

A possible solution could be the regenerative medicine that consists in the concept of regenerating rather than the simply replacing of damaged tissues,

using specific materials able to stimulate tissue regeneration (Ref) It was demonstrated that Nano-Hydroxyapatite-coated silk scaffolds improved dental regeneration by stimulating both PDL cells and DPSCs in dogs. Again, the Bioactive glass (BG) nano-filled composite resin showed an effect on the proliferation of human DPSCs, increasing mineralization and the expression of odontogenic-related protein. Moreover, an *in vitro* cell culture study performed on human DPSCs demonstrated that scaffolds made of nanofibrous gelatin/silica BG showed better odontogenic differentiation potential compared to scaffolds without bioactive glass. Another *in vitro* study proved the efficacy of nano-carbonate apatite in preventing the restaining after the bleaching of the dental enamel [111]. However, regenerative materials are still at the stage of experimental design and their performances are mainly confined to preclinical phase.

2.4. Bio-organic and inorganic small molecules for tissue regeneration

Dental regenerative materials, in the form of scaffolds, membranes or nanoparticles, can be loaded with bio-organic or inorganic small molecules, to promote tissue regeneration. Among the organic molecules, cytokines and growth factors are the most commonly used, since they are soluble secreted proteins capable of affecting a variety of cellular processes fundamental for tissue regeneration [112].

Dentin is a rich source of growth factors and bioactive molecules which are liberated when dentin matrix is demineralised/solubilized by bacterial acids during a carious attack or by certain dental materials like $Ca(OH)_2$, MTA (Mineral Trioxide aggregate) and acid etchants used during some restorative

treatment [113]. A sophisticated approach includes the use of growth factors, in particular the bone morphogenetic protein (BMPs), added to dental resin to facilitate reparative dentin formation stimulating viable endogenous pulp tissue cells.

BMPs, a group of regulatory glycoproteins members of the transforming growth factor-beta superfamily, play a critical role in tooth morphogenesis, along with other growth and differentiation factors (GDFs) [114]. In particular, BMPs 2, 4, and 7 are expressed in dental epithelium, and recombinant BMPs 2 and 4 can be used as a substitute for dental epithelium in inducing differentiation of mesenchymal stem cells [115]. Recent studies highlight the tissue regeneration capability of BMPs, loaded in different materials as scaffold [116], sponge [117] and nanoparticles [118]. The synthesis of injectable poly lactide-co-glycolide (PLGA) and polycaprolactone (PCL) nanoparticles loaded with recombinant human BMP-2 [119] demonstrated dental tissue regeneration potential of BMPs released from materials. The use of growth factor in the development of commercial regenerative material is limited by their short half-life (they are easily denatured/inactivated), the high costs associated with the application of their recombinant forms [120], their potential diffusion in other tissue far from the application site and their low cell- targeting specificity [110]. Moreover, the use of growth factors (and consequently the overexpression of their receptors) in border-line situations, can result in an increased risk of developing malignancies. Many tumors used growth factors to stimulate angiogenesis, mitogenesis, cell differentiation and metabolism [121]. Indeed, a related difference between normal cells and cancer cells is that for some cell types the availability of serum growth factors is the principal determinant of their proliferative capacity in culture [122].

For these reasons, research attention has focused to the use of inorganic bioactive molecules able to induce tissue regeneration. In particular, calcium phosphate and fluoride are the most promising agents [123].

The inorganic part of hard tissues of mammals consists of calcium phosphate (CaP) [124]. The release of Ca at the pulp enhances the activity of bone-associated proteins and pyrophosphatase, which helps to maintain dentin mineralization and the formation of new dentin bridging. It was demonstrated that synthetic octacalcium phosphate (OCP) enhanced reparative dentine formation during the early stage *via* downregulation of Runt-related transcription factor 2 (Runx2) expression in pulp cells. Indeed, Runx2 is involved in tooth development prior to the bell stage, but not in later stages [125]. In addition, Qader *et al*. evaluated the influence of biphasic calcium phosphate (BCP) scaffolds of hydroxyapatite/beta-tricalcium phosphate with controlled pore size, on the human dental pulp cells differentiation toward odontoblast phenotype. The high expression of collagen type I alpha 1, dentin matrix protein-1, and dentin sialo-phosphoprotein demonstrated that this scaffold can support human dental pulp cells differentiation for dentin tissue regeneration [126]. Skrtic *et al*. synthetized amorphous calcium phosphate (ACP)-based resins to investigate their effect on odontoblast-like cells. Exposing to oral fluids, this resins released Ca and PO_4 ions in a sustained manner, reaching supersaturation conditions favourable for the regeneration of tooth [127]. Indeed, CaP and its derivatives have been widely and successfully used as scaffolds with osteoblasts for bone tissue regeneration. A recent *in vivo* study showed the repair of defects in the pulp chamber floor of premolars, using deciduous pulp stem/progenitor cells from pig primary teeth associated with a beta-phosphate-tricalcium scaffold. After 16 weeks, complete closure of the defect by a mineralized barrier was observed,

demonstrating that the association of stem cells with the scaffold resulted in adequate dentin regeneration [128].

Another inorganic ion able to influence dental pulp stem cells behaviour is fluoride. Fluorine is the world's 13th most abundant element and constitutes 0.08% of the Earth crust. Among the other elements fluorine is the most electronegative. Fluoride (F^-), the inorganic anion of fluorine, is also extensively diffused, since air, soils, rocks, and water can contain F^- [129]. It is well known that this anion can promote osteogenesis and activate osteoclast (responsible cells for the dissolution and absorption of bone), influencing bone metabolism directly or indirectly by enhancing calcium influx [130]. Similarly, F^- influence odontogenesis and it was demonstrated that fluoride can affects odontoblast gene expression *in vitro*, without inducing cell stress or apoptosis if fluoride solution concentration not exceed 1mM [14]. In fact, fluoride positive effects on odontoblasts are concentration and time depending. Peng et al reported that exposure of odontoblasts to NaF 4 mM for 24 h induced apoptosis via c-Jun N-terminal kinases (a member of the mitogen-activated protein kinase family) dependent mitochondrial pathway [131]. In addition, fluoride influences odontogenesis by stimulating type I collagen deposition in a dose-dependent manner, since high concentrations induce the opposite effect. In the early stages of tooth germ development, the formation and mineralisation of dentin is regulated by events occurring within the extracellular matrix and type I collagen, phosphoproteins such as phosphophoryn, osteopontin, osteonectin, and dentin sialoprotein represent the major structural proteins of the extracellular matrix [132]. Indeed, Mei *et al* demonstrated that silver diamine fluoride inhibited demineralisation and preserved collagen from degradation in demineralised dentin [133].

A histopathological study confirmed that fluoride releasing Type VII-glass ionomer and silver diamine fluoride did not induce any inflammation/necrosis in the pulp, allowing good tertiary dentin deposition by odontoblasts [134]. Moreover, R.J Waddington *et al* indicated that fluoride influence mineralization patterns, inducing changes into proteoglycan (PG) structure synthesised within the dentine–pulp complex *in vitro*. Indeed, proteoglycans play important structural and metabolic functions in the formation of mineralised tissues [135].

Fluoride represents one of the most studied small inorganic molecule for tissue regeneration since seems to affect cell migratory response to chemotactic factors. Recent studies on human osteoblast-like cells, exposed to released fluoride from fluoridated hydroxyapatite granules, show that F^- can influence cell migratory response to chemotactic factors in a dose-dependent manner [136]. Probably, the migratory effect of fluoride is related to its capability to activate G protein signal molecules in various cell lines, stimulating signaling cascades that result in cell shape changes and formation of a migrating front [137]. In fact, fluoride with aluminum ions (Al), forms AlF complexes that binds γ-phosphate of GDP and create a GDP-AlF complexes that mimic GTP molecules, inducing G protein active configuration [138]. However, fluoride role in DPSCs migration remains unclear, since the network of material tissue interactions is complex and comprises a wide range of issues [139].

3. Experimental design: restorative plus regenerative dentistry.

The choise: layered double hydroxide as filler in dental composite resin

Nowadays, the most adopted composites in the clinical practice are the glass ionomer cements containing fluoride. Unfortunately, GICs have limited pharmacokinetic properties due to a burst release of the active principle in the first 24 hours that can lead to cytoxicity [7]. Moreover, such materials are not able to recruit stem cells for regenerating damaged tissues due to the fact that DPSCs require a controlled fluoride release kinetic, both for time and concentration. Then, the actual dental restorative materials promote tooth remineralization using the remained substrate of MID surgery and are not able to promote the formation of new tissues through the recruitment of DPSCs. On the other hand, regenerative dental materials still remain at clinical stage and tissue regeneration represents a long process. Thus, this thesis project is aimed to the synthesis of a “restorative plus regenerative material” that overcomes the limitations of actual available materials.

The experimental design (fig.11) was planned in order to obtain a new fluoride restorative/regenerative dental material with improved bioactivity and adequate mechanical properties. The project idea was to take advantage from a delivery of low amount of fluoride for a long period of time, able to promote both DPSCs migration and differentiation into odontoblasts, avoiding the cytotoxic effect of higher fluoride concentration. This goal can be obteined by using fluoridated-Layered double hydroxide (LDH) as filler, to improve fluoride release kinetic.

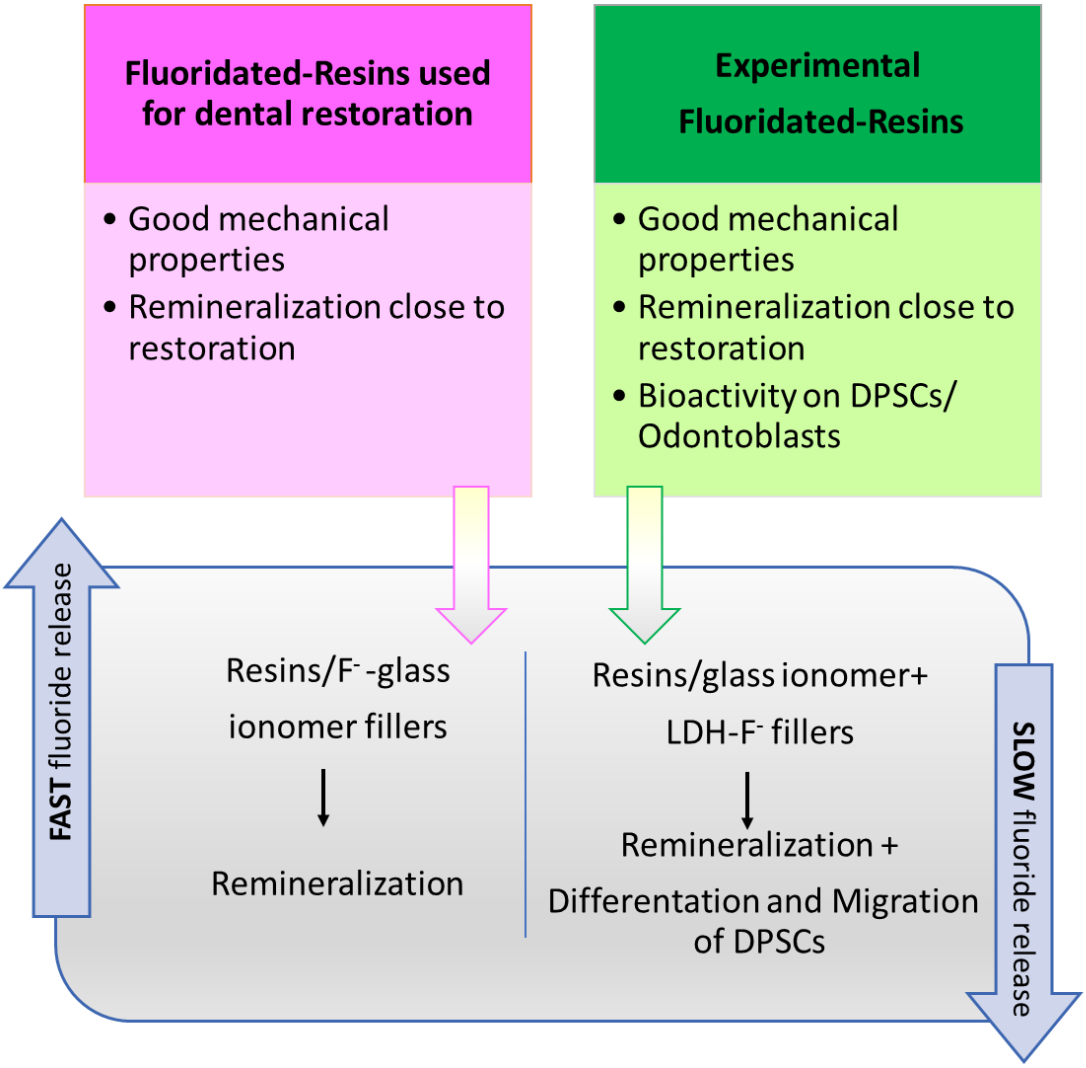

Fig.11: Scheme of the experimental design.

3.1. Layered double hydroxide (LDH) as new fillers in dental composite resins

Hydrotalcites, also defined anionic clays or layered double hydroxide (LDH), are magnesium aluminium-hydroxycarbonate naturally occurring in lamellar warped form. They were discovered in Switzerland in 1842, but their molecular formula, $Mg_6Al_2(OH_{16})(CO_3) \cdot 4H_2O$, was published for the first time only in 1915 by Manasse. In 1942, Feitknecht introduced the term "double-layer compound" assuming that the synthesized compounds with hydrotalcite structure are constituted of intercalated hydroxide layers. This hypothesis was confirmed by Allmann and Taylor who demonstrated that the cations are co-located in the layers while carbonate anions are intercalated together with water molecules. Overall, the structure of hydrotalcite is comparable to brucite, ($Mg(OH)_2$), where each Mg^{2+} ion is bound to six OH^- ions with octahedral coordination.

In particular, the octahedra share an angle, forming layers organized on top of each other and bound together by hydrogen bonds. If part of Mg^{2+} ions is substituted by trivalent ions of suitable size, such as Al^{3+}, layers become positive and this charges are balanced by intercalated anions (the most common is carbonate). Water molecules in interlayers are binded to layers and anions by hydrogen bonds (fig.12) [140]. New combinations of LDH are obtained changing metals during synthesis processes. Below are listed the most used:

- Divalent Metals = zinc (Zn^{2+}), cobalt(Co^{2+}), nickel (Ni^{2+}), manganese (Mn^{2+})
- Trivalent Metals =chromium (Cr^{3+}), iron (Fe^{3+}), vanadium (V^{3+}), cobalt (Co^{3+}) e gallium (Ga^{3+}) [141].

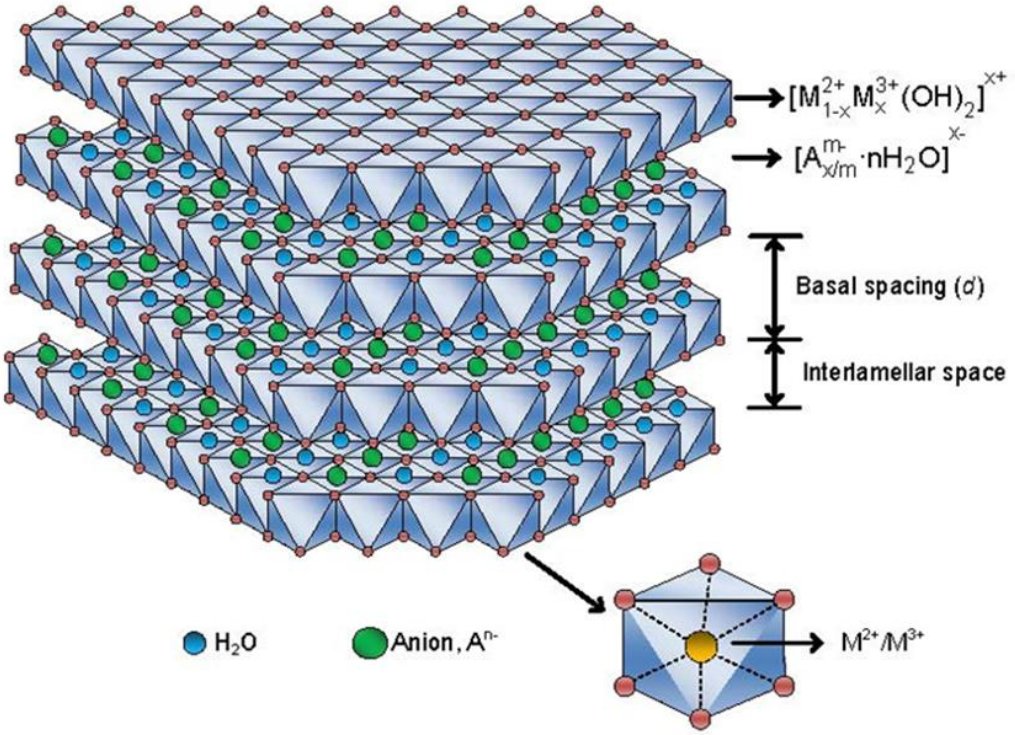

Fig.12: Schematic representation of LDH structure (Reproduced from Tronto et al., 2013 [142]).

The loading of anions interposed between lamella sheets is carried out by two simple methods: ion-exchange or co-precipitation [140].

LDH/ polymer/anion complexes were firstly evaluated for pharmaceutical application, in particular for anti-cancer drugs [143-145], as delivery carrier [141]. In fact, the incorporated negative charged molecules can be drugs, bioactive anions or nucleic acids, substances able to elicit a therapeutic action.

Moreover, potential fillers can be composed also of nanosized LDHs (NPs-LDH). Several studies have demonstrated nano-LDHs biocompatibility and activity. In fact, observations on Human Osteosarcoma Cells confirmed LDH biocompatibility and defined its intracellular trafficking. In particular, microscopy analysis showed the size dependence of the cellular uptake of LDH nanoparticles. Specifically, 50 nm NPs-LDH followed lysosomal degradation

pathway, whereas 100 nm NPs-LDH were not degraded and were probably exocytosed from the Golgi [146].

Mouse Motor Neuron uptake studies confirmed the importance of LDH nanoparticles dimensions in cellular localization. Two different LDH-NPs were analyzed using fluorescein isothiocyanate labeling: 20 nm CO_3-LDH and 180 nm NO_3-LDH. Confocal laser microscopy results showed that 20 nm LDHs-NPs were localized in nucleus, while 180 nm LDH-NPs in the cytoplasm [147].

The release kinetics is a further characteristic that can be optimized in loaded LDHs. Different chemical compositions of LDHs exhibit distinct adsorption and release kinetics of loaded drugs. For example, the concentration of intercalated fluoride is higher for clays consisting of Mg/Al, compared with Ni/Al or Zn/Al, due to the greater atomic weight of Zn and Ni and the consequent lower capability of anion exchange, compared to Mg. Molar ratio Mg/Al was also investigated, since it may influence layers charge density and their anionic adsorption capability. In particular, 2:1 molar ratio shows a higher charge density and fluoride retention than 3:1 and 4:1 molar ratios [148]. Moreover, LDH with metal molar ratio 2: 1 adbsorbs fluoride more efficiently in nitrate form (LDH-NO_3) compared to those in chlorinated form (LDH-Cl^-) according to second order kinetic. However, the F^- ions in the resulting LDH-F can be anion-exchanged in solutions containing NO_3^- or Cl^- (fig.13) [149]. Thus, in presence of acidic microenvironment induced by cariogenic bacteria, the LDH anionic exchange between fluoride and nitrate could be facilitated increasing fluoride release concentration.

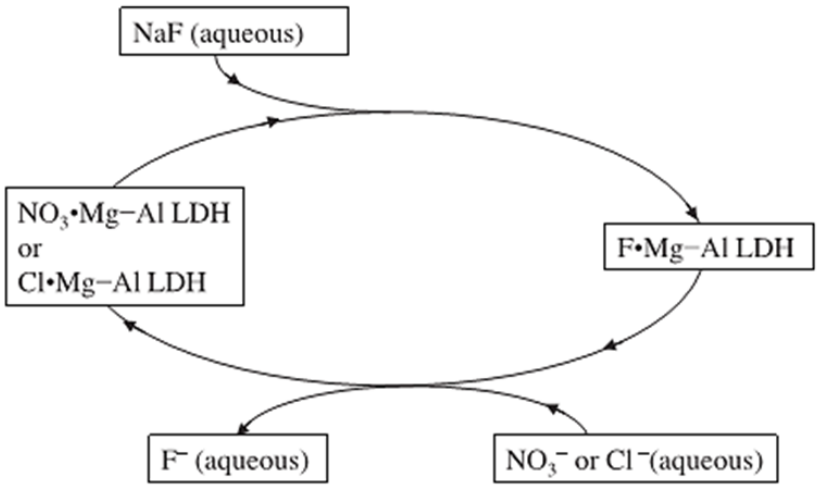

Fig.13: the recyclability of LDH based on anion exchange (Image proposed by Kameda et al 2015 [149]).

Indeed, other crucial design parameters are the pH of the solution containing the anions to load and that of the fluid in which their release occurs. Strongly basic pH leads to a wide presence of OH^- ions that competes with F^- ions during anionic exchange in loading/release process while acid pH triggers LDH layers degradation, releasing the anion in few minutes. For example, LDH release capability in acid conditions is used in enteral administration (gastric fluid pH is 1.2) [150].

LDHs release capability was also investigated according to their concentration in resin in order to modulate the release kinetics. Hypothetically, concentration optimization must be related with appropriate LDH delamination, to guarantee anionic exchangers accessibility and to confer a right degree of rigidity to the resin. Usually, nano-filler concentration inside the resin varies from less than 3% w/w to 20% for larger size ones [151]. Indeed, Rojas and co-workers demonstrated that low hydrotalcites concentrations induce an uncontrolled release, due to excessive layers slip. In fact, low

hydrotalcites concentrations compressed resins in platelets showed an initial release without classical burst effect and uncontrolled kinetics [152].

Finally, LDHs as fillers were also exploited for industrial composites due to their capability to improve the mechanical properties (rigidity and flexibility) of the material in which they are inserted. In addition, industrial LDH composites are also able to protect the material from degradation triggered by UV rays, since they are absorbed between the layers [153]. Such feature is interesting in dentistry due to the widespread use of light-curing resins in restoration.

For all these properties, LDH has been chosen as filler in our experimental dental composite resins as well as to exploit the possible fluoride biological activity on DPSCs.

4. Materials and Methods

4.1. Preparation of fluoride layered double hydroxide (LDH-F)

The LDH in the nitrate form $[Mg_{0.65}Al_{0.35}(OH)_2](NO_3)_{0.35}0.68H_2O$ (LDH-NO_3) was prepared by ammonia precipitation to exclude CO_3^{2-} ions from the interlayer region. In a typical preparation, a mixed metal nitrate solution (1 M) containing the stoichiometric requirement ratio of Mg^{2+} and Al^{3+} ions, was added dropwise to a solution of ammonia (1 M) with vigorous stirring at ambient temperature (25–27°C). containing ten times the stoichiometric requirement of NO^{3-} ions. The resulting slurry was stirred for 1 h then hydrothermally treated (140°C, 24 h; 50 % filling) in a Teflon-lined autoclave. The solid obtained was separated from the supernatant by centrifugation, washed several times with decarbonated, deionized water until the pH of the wash was neutral, and dried in an oven at 60°C.

To obtain fluoride-intercalated layered double hydroxide (LDH-F), 500 mg of LDH-NO_3 was equilibrated with a decarbonated aqueous solution (10 ml) of 0.25 mol/dm^3 fluoride sodium salt (NaF). The exchang reaction was performed for 48 hours under stirring and nitrogen flow at room temperature. The molar ratio between F^- and NO_3^- was 1.3 as reported by Costantino et al. [154, 155].

The obtained white solid was separated by centrifugation, washed three times with CO_2-free deionized water and finally dried at room temperature over a saturated sodium clorure (NaCl) solution (75% of relative humidity, RH). The final product (LDH-F) has the formula $[Mg_{0.65}Al_{0.35}(OH)_2](F)_{0.35}\ 0.8\ H_2O$.

4.2. Preparation of resin containing LDH-F

The LDH-F (with mass fraction of 0.7, 5, 10 and 20%) was added into a commercial light-activated restorative material (RK) provided by Kerr s.r.l.

(Italy), which consisted of bisphenol-A glycidyl dimethacrylate (Bis-GMA), tri-ethylene glycol dimethacrylate (TEGDMA), camphorquinone (CQ), ethoxylated bisphenol A dimethacrylate (EBPADMA) and glass fillers. The samples are coded RK-Fx, where x is the percentage by weight of the inorganic solid LDH-F in the resin RK. RK-FG10 was prepared by adding an amount of fluoride-glass filler (FG; Kerr s.r.l.) to obtain a final fluoride concentration of about 0.9% by weight in RK. RK composite resin was used as a control. Specimen disks 14 mm in diameter and 1 mm thick were fabricated using steel molds. The composite obtained were cured by photo-polymerization using a visible light curing unit (Optilux 380, distributed through KERR, USA; irradiated diameter: 11 mm) with an irradiation time of 120 s. During the experiment, the light intensity was maintained at 550 mW/cm^2.

Before incubation with cells, all the RK-Fx resins were gas sterilized using ethylene oxide. Second passage DPSCs were seeded at a concentration of 1 x 10^4 cells/well directly onto RK-Fx resins (14 mm in diame-ter) placed in 24-well plates (Falcon). The non-adherent cells were removed by washing three times in medium at 24 h.

4.3. Characterization and evaluation

4.3.1. X-RAY POWDER DIFFRACTION (XRPD)

XRPD patterns were recorded, in reflection, with an automatic Bruker diffractometer (equipped with a continuous scan attachment and a proportional counter), using the nickel filtered Cu Ka radiation (l = 1.54050 A°) and operating at 40 kV and 40 mA, step scan 0.058 of 2u and 3 s of counting time.

4.3.2. FOURIER TRANSFORM INFRARED ANALYSIS (FT-IR)

Infrared absorption spectra were obtained by a Bruker spectrophotometer, model Vertex 70, with a resolution of 4 cm^{-1} (32 scans collected).

4.3.3. DYNAMIC-MECHANICAL ANALYSIS

Dynamic-mechanical properties of the samples were performed in triplicate with a dynamic mechanical thermo-analyzer (TA instrument-DMA 2980). The samples were tested by applying a variable flexural deformation in dual cantilever mode. The displacement amplitude was set to 0.1%, whereas the measurements were performed at the frequency of 1 Hz. The range of temperature was -50 to 150 °C, scanning rate of 3 °C/min.

4.3.4. FLUORIDE RELEASE STUDY

Weighed disks of all samples were placed at 37°C under magnetic stirring in physiological medium and artificial saliva medium (SAGF, 15 mL) [156]. SAGF was prepared from calculated amounts of chemicals supplied by Sigma-Aldrich (Milan, Italy), according to the procedure described in the literature [157, 158]. After the time intervals (every hour for 8 h, then every day for 10 d, and then every week for 3 wk), the free fluoride ion concentration (ppm) was determined using an ion chromatograph (DX 100, Dionex; Thermo Scientific, Milan, Italy) with suppressed conductivity as previously described (McCabe et al. 2002 [159]). The analysis was done in triplicate and the values averaged.

4.4. Cell Isolation and characterization

Human dental pulp stem cells (DPSCs) were isolated from normal, non-carious impacted third molars from 10 adults (18–22 years of age) as previously described [160]. The teeth were obtained in compliance with Italian legislation (including informed consent and Institutional Review Board approval of the protocol number 7413). The teeth were cleansed of external organic and inorganic debris with 70% isopropanol. In cases where the teeth were incompletely developed, the apical papilla was removed to prevent contamination [161]. Teeth were sectioned longitudinally; pulpal tissue was gently removed with tissue forceps and cut into small fragments. The tissue was then placed in Phosphate buffered saline (PBS) prior to enzymatic digestion with 3 mg/ml of collagenase type I and 4 mg/ml dispase for 30 min at 37°C. The digested mixtures were passed through a 70-mm cell strainer (Falcon, Italy) to obtain single-cell suspensions and centrifuged for 10 minutes at 1,800 rpm. Cells were seeded onto six-well plates and cultured in Growth medium [GM, α-minimum essential medium (α-MEM) supplemented with 15% FBS, 2 mM L-glutamine, 100 mM L-ascorbic acid-2-phospate, 100 U/ml penicillin-G, 100 mg/ml streptomycin, and 0.25 mg/ml fungizone (Hyclone, Italy)] and maintained in 5% CO_2 at 37°C.

4.5. Colony-forming ability and proliferation of DPSCs

Clonogenic assay or colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The colony is defined to consist of at least 50 cells. The assay essentially tests progenitor cells ability to undergo "unlimited" division, to differentiate and proliferate.

Colony Forming Unit-Fibroblast (CFU-F) assays were performed by plating third to sixth passage DPSCs isolated from 10 donors in a 6-well plate at 50 and 100 cells/well in GM. After 14 days in culture at 37°C in a humidified atmosphere containing 5% CO_2, the cells were washed twice with PBS, fixed with ice-cold methanol for 5 min at room temperature, and stained with 0.3% crystal violet for 15 min. The cells were washed with distilled water and the number of colonies was counted. Colonies greater than 2 mm in diameter were enumerated.

Proliferation assay was performed by seeding DPSCs in a 6-well plate at 4 x 10^4 cells/well in duplicate, and incubating them in GM for 14 days at 37°C in a humidified atmosphere containing 5% CO_2. At days 3, 6, 10 and 12, the cells were harvested by trypsin-EDTA treatment, washed in GM and centrifugated at 1,500 rpm for 10 min. The pelleted cells were then counted with a hemocytometer and their viability determined by the trypan blue dye exclusion test.

4.6. Effect of fluoride on DPSCs proliferation

To assess the proliferation rate of the cells in presence of different concentrations of fluoride, total DNA content (n = 3) was measured by a PicoGreen dsDNA quantification kit (Molecular Probes, Italy). Cells were seeded at a density of 4 × 10^3 cells/well in a 96-well plate, and after 24-h incubation, medium was replaced with fluoride-medium (fluoride final concentrations of 0.5, 1.0, 2.0 and 5.0 ppm) for a further 48 h. Then the cells were washed twice with a sterile phosphate buffer saline (PBS) solution and transferred into 1.5 ml microtubes containing 1 ml of ultrapure water (Eppendorf, Italy). Samples were incubated for 1 h at 37°C in a water bath,

subjected to a freeze-thaw cycle and sonicated for 15 min before DNA quantification. Then 100 µl of PicoGreen working solution was added to 100 µl of supernatants of the samples. Triplicates were made for each sample or standard. The plate was incubated for 10 min in the dark and fluorescence was measured on a microplate reader (Cytation 3, ASHI) using an excitation wavelength of 490 nm and an emission of 520 nm. A standard curve was created and sample DNA values were read off from the standard graph.

4.7. Magnetic-activated cell sorting (MACS)

To obtain STRO-1^+ stem cells, hDPSCs were directly sorted from pulp cell cultures at passage 3 with immunomagnetic beads Dynabeads according to the manufacturer's protocol (Life Technologies, Milan, Italy). In particular, approximately 5×10^6 cells were incubated with mouse anti-human STRO-1 at 4°C for 30 minutes, washed with PBS/5% bovine serum albumin (BSA), and resuspended with rat anti-mouse IgM-conjugated Dynabeads on a rotary mixer for 60 minutes. After washing, bead-positive cells were separated with a magnetic particle separator and subsequently placed into 75 cm^2 culture flasks. Immunosorted DPSCs were cultured, passaged in the routine culture media (α-MEM) at 37°C in 5% CO_2, and observed under the phase-contrast inverted microscope (Olympus). Approx 5% of DPSCs in the primary cells can be harvested by STRO-1-mediated MACS method.

After cell sorting, each of the following experiments was performed in triplicate on pooled STRO-1–sorted cells (STRO-1^+ cells).

4.8. Alkaline Phosphatase activity

Alkaline phosphatase activity is a typical marker for early odontoblastic differentiation. ALP activity was assessed as reported by Wang et al [162] on DPSCs and STRO-1^+ cells cultured for 28 days in GM and osteogenic-induction medium (OIM, α-MEM, 10% FBS, 100 nM dexamethasone, 10 mM sodium β-glycerophosphate, 100 mM L-ascorbic-acid-2-phosphate, 2 mM L-glutamine, 100 U/ml penicillin-G, 100 mg/ml streptomycin, and 0.25 mg/ml fungizone). At predetermined days, the cells were scraped into cold PBS, sonicated in an ice bath and centrifuged at 1500 g for 15 min. ALP activity was measured in the supernatant using p-nitrophenyl phosphate as a phosphatase substrate. The absorbance was measured at 405 nm recording data every 5 min for 30 to 60 min. For end-point the reaction were incubated for 30-60 min with 50 μL of Stop Solution into each well before reading. The amount of ALP in the cells was normalized against total protein content.

4.9. Cell migration of STRO-1^+ cells by transwell chemotaxis assay

The cell migration assay was performed using a modified Boyden Chamber method. Boyden Chamber consists of a cylindrical cell culture insert nested inside the well of a cell culture plate. The insert contains a polycarbonate membrane at the bottom with a defined pore size usually coated with extracellular matrix compomponents such as collagen I, fibronectin etc. Cells are placed in the upper compartment and are allowed to migrate through the pores of the membrane into the lower compartment, in which chemotactic agents are present. STRO-1^+ cells cultured for 7 days on RK-Fx and RK-FG10 resins were trypsinized and plated on the upper side of the filter, that was

precoated for 2 h at 37°C with α-MEM containing 1% FBS and 0.25% (w/v) bovine serum albumin (BSA) to facilitate cells' initial attachment. The medium in the lower chambers was replaced with α-MEM containing transforming growth factor β1 (TGF-β1, 150 ng/mL) or stromal cell–derived factor 1α (SDF-1, 150 ng/mL), and the migration of STRO-1$^+$cells was monitored. After 24 h, the cells that had migrated to the lower side of the filter were fixed with 4% paraformaldehyde for 30 min, stained with crystal violet for 20 min, and counted under a microscope in 7 predetermined fields. All experiments were independently repeated3 times.

4.10. Odontogenic-related gene expression of STRO-1$^+$cells by real-time polymerase chain reaction

Real-time Polymerase Chain Reaction permits simultaneous amplification and detection of specific DNA-sequences. The amount of product formed was monitored during the course of the reaction through the fluorescence of the probes that was introduced into the mix. The number of amplification cycles required to obtain a particular amount of DNA molecules was registered [163].
To evaluete the expression levels of odontogenic-related genes, corresponding RNA were quantified in form of cDNA. Indeed, total RNA was extracted from STRO-1$^+$ cells seeded in the presence of resins for 28 days, using TRIzol reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions. Total RNA (0.2 μg) was first treated at 37°C for 30 min with DNase (Promega, Milan, Italy) and then subjected to reverse transcription (RT) with 0.4 μg random hexamers and 20 U AMV reverse transcriptase (Promega) in a 25-μL reaction mixture at 42 °C for 1 h. The resulting cDNA mixture was amplified by real-time polymerase chain reaction (PCR) using specific primers for osteocalcin (*ocn*),

matrix extracellular phosphoglycoprotein (*mepe*), dentin sialophosphoprotein (*dspp*), dentin matrix protein 1 (*dmp1*), and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) as listed in Table 3.

Genes	Forward primers (5'-3')	Reverse primers (5'-3')	Annealing
ocn	CCACCGAGACACCATGAGAG	CCATAGGGCTGGGAGGTCAG	66°C
mepe	GGTTATACAGATCTTCAAGAGAGAG	GTTGGTACTTTCAGCTGCATCACT	60°C
dspp	AGAAGGACCTGGCCAAAAAT	TCTCCTCGGCTACTGCTGTT	60°C
dmp1	TGGGGATTATCCTGTGCTCT	TACTTCTGGGGTCACTGTCG	56°C
gapdh	ACATGTTCCAATATGATTCCA	GGACTCCACGAGCTACTCAG	60°C

Table 3: Specific primers used in RT-PCR

Real-time PCR assays were run on an Opticon-4 machine (Bio-Rad, Milan, Italy). The reactions were performed using 25µl of SYBR Green PCR Master mix per reaction (Invitrogen). The PCR conditions were as follows: AmpliTaq Gold DNA Polymerase (Life Technologies) activation for 10 min at 95 °C and 40 cycles at 95 °C (denaturation) for 15 s and 60 °C (annealing/extension) for 1 min. All reactions were run in triplicate and were normalized to the house- keeping gene, *gapdh*. Relative differences in the PCR results were calculated using the comparative cycle threshold (CT) method. The variations in gene expression are given as arbitrary units.

4.11. Statistical Analysis

All quantitative data are presented as the mean ± SD. Each experiment was performed at least 3 times. Student's *t* test was used for the fluoride release. Statistical analyses for the cytotoxicity test, cell migration assay, and quantitative real-time PCR were performed by 1-way analysis of variance (ANOVA) with Bonferroni's post hoc test.

5. Results and Discussion

5. 1. Structural analysis of fluoride hydrotalcite (LDH-F)

Structural information was obtained by X-ray analysis (XRPD) and FT-IR absorption spectroscopy. Fig. 14a shows the diffraction patterns of the pristine hydrotalcite in nitrate form (LDH-NO_3) and the intercalated fluoride form (LDH-F). As a result of the NO_3/F exchange, the X-ray reflection due to the interlayer distance (d003 = 0.902 nm) and positioned at 2θ = 9.9° (LDH-NO_3) moves at much higher angles, 2θ = 11.68°, corresponding to an interlayer distance of 0.757 nm (LDH-F). The decrease of the interlayer distance was due to the intercalation of the fluoride anion with smaller dimension than the nitrate anion [164]. The second and third patterns of LDH-F spectra correspond to the higher harmonics of the interlayer distance. All the peaks are sharp, and this indicates an ordered accommodation of the inorganic anion within the interlayer regions. Moreover, the X-ray reflections of the nitrate form are absent, and this indicates that the nitrate ions left out of the exchange were solubilized in the interlayer region of the fluoride intercalate. Fig. 14b shows the FT-IR absorbance spectra for the LDH-F sample. The intercalation of fluoride ions into LDH is represented by the characteristic band at frequency 1385 cm^{-1} [164, 165]. Adsorption bands around 550 and 680 cm^{-1} can be attributed to the Mg–OH and Al–OH translation modes, respectively [166]. The adsorption band around 3460 cm^{-1} can be attributed to the OH group stretching due to the presence of hydroxyl groups of LDH or co-intercalated water molecules, or both [167].

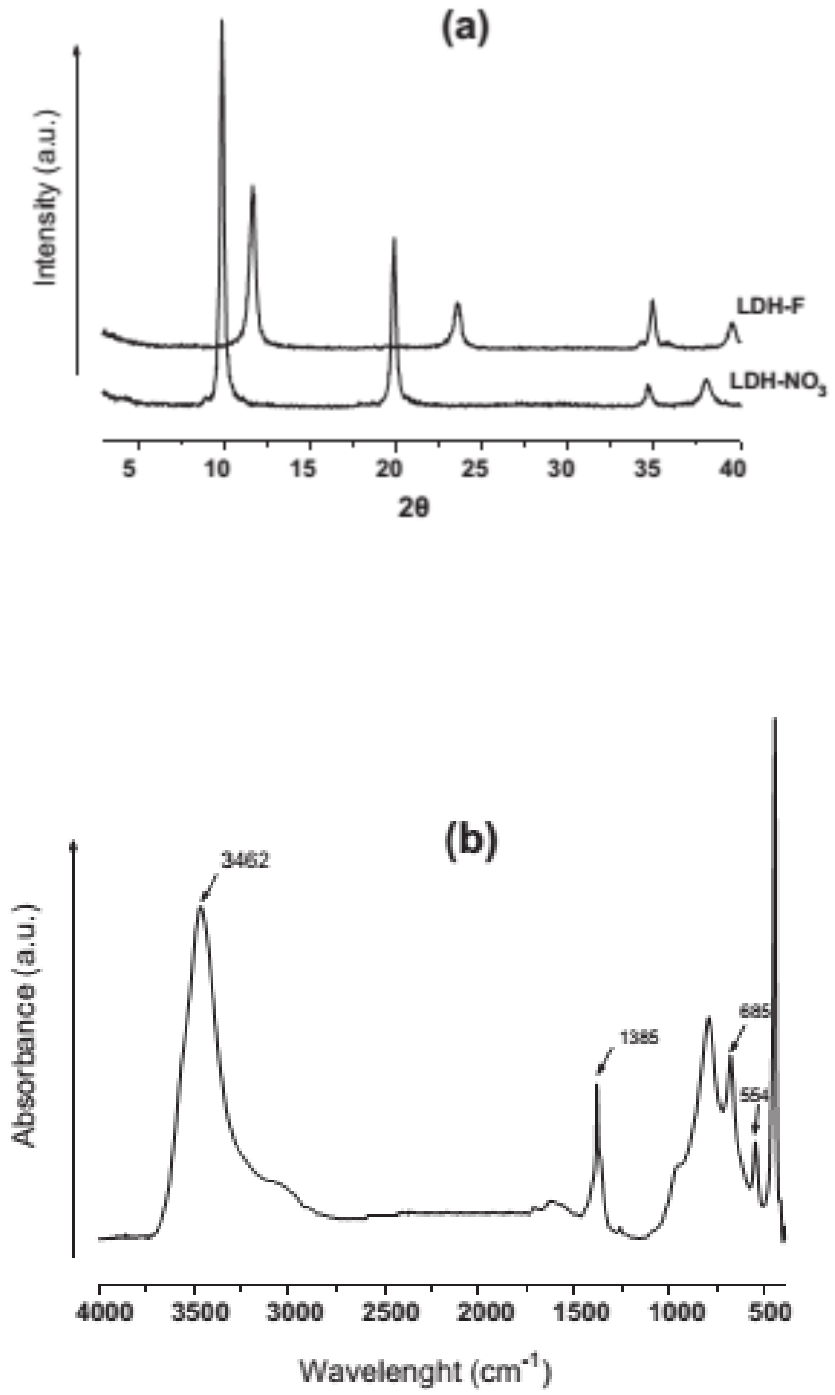

Figure 14: (a) XRD patterns of LHD-NO_3 and LDH-F and (b) FT-IR spectra of LDH-F.

5.2. Incorporation of LDH-F into the dental resin

5.2.1. STRUCTURAL INVESTIGATION

The incorporation and dispersion of the inorganic solid into the dental resin was investigated by X-ray analysis. The diffractograms of the LDH-F, the pure resin and the composites with LDH-F are displayed in Fig. 15. The broad pattern in Fig. 2b is attributed to the reflection of amorphus RK, while the diffraction spectra of RK/LDH-F composites (Fig. 2 c–f) show characteristic reflections of LDH-F powders at 2θ = 11.68° and 23.6 besides the broad reflection of pristine RK, increasingly evident starting from the sample at 5% of concentration (RK-F5). In particular, the basal peak at 11.68° of 2θ, absent at low concentration, appears in the sample RK-F5 (c) and then increases on increasing the inorganic concentration. This suggests that the clay is delaminated and well dispersed up to 5% of concentration, whereas at higher concentrations large clay tactoids are present in the sample. The X-ray data therefore indicate a morphology with micro-domains of LDH-F at concentrations higher than 5%.

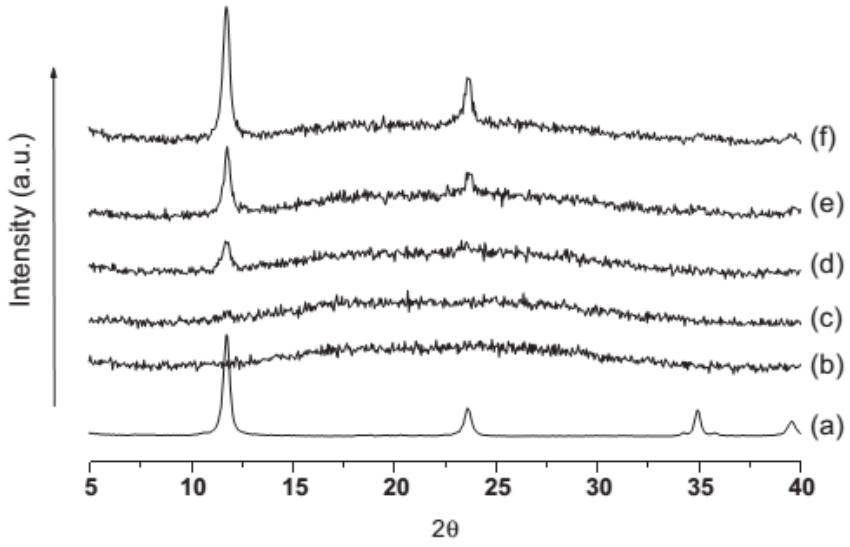

Figure 15: XRD patterns of (a) LDH-F, (b) RK resin, (c) RK-F0.7, (d) RK-F5, (e) RK-F10, and (f) RK-F20.

5.2.2. MECHANICAL PROPERTIES

The mechanical properties were investigated in a wide range of temperatures by performing a dynamic mechanical analysis able to detect either the elastic modulus and the tan δ in the investigated range of temperature. Fig. 16 shows the elastic modulus for the pristine resin and the resin containing 5, 10 and 20 wt.% of LDH-F. The study of the mechanical properties in a wide temperature range demonstrated that the values of the elastic modulus of the resins containing the fluoride inorganic solid (RK-Fx) increased compared with the resin RK. This increase, which was evident after the glass transition temperature, was observed at different temperatures and for different compositions.

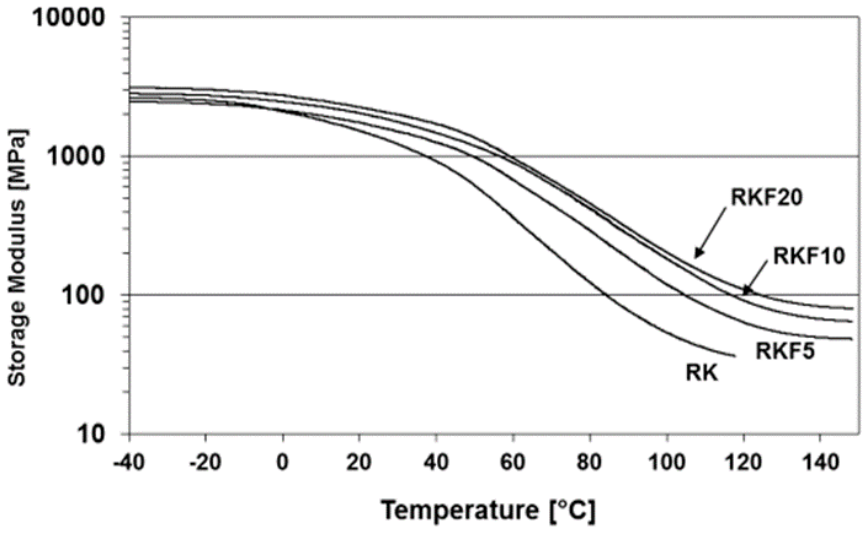

Figure 16: Storage modulus (MPa) *versus* temperature (C°) of RK, RK-F5, RK-F10, and RK-F20.

Figure 17 (a-b) reported the comparison of the storage moduli at three different temperatures (0°C, 37°C, 50°C) *versus* the values of the glass transition temperatures for RK-Fx resins. The storage moduli of the composite resins are consistently higher than the pristine resin, in particular at 37°C. The observed reinforcement increased with the increase in the filler concentrations. As expected for a composite system, the deformation at breaking of the composite resin was found slightly lower than the pristine resin. However, since the stress is increasingly higher in the composites, the toughness remained almost unchanged.

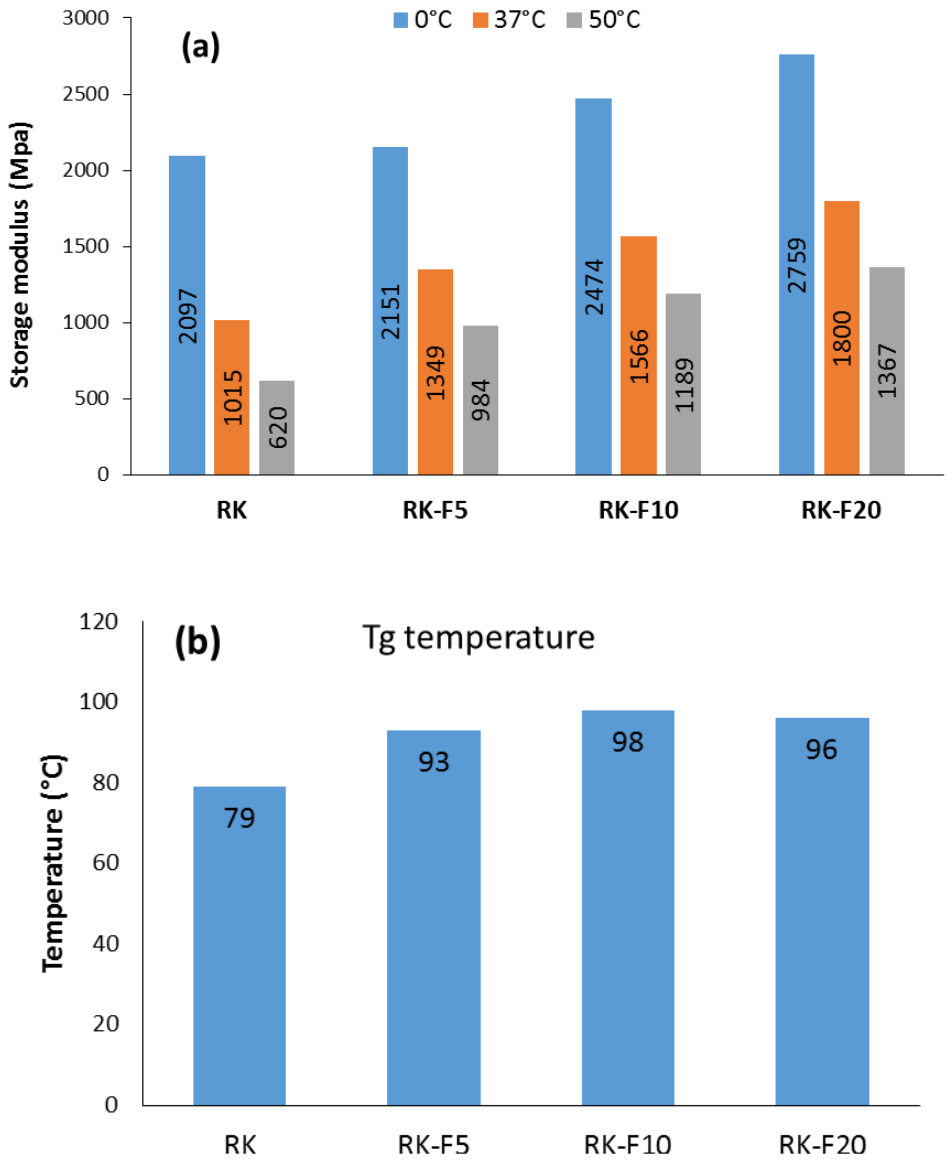

Figure 17: (a) Storage moduli (MPa) at 0°C, 37°C and 50°C and (b) glass transition temperature (°C) for the pristine resins and its composites.

Moreover, similar results were obtained when compared RK-F10 with the same commercial dental resin supplemented with a fluoride-glass filler (RK-FG10) (Figure 18). In particular, the deformation at breaking of the RK-F10 was slightly lesser than RK-FG10.

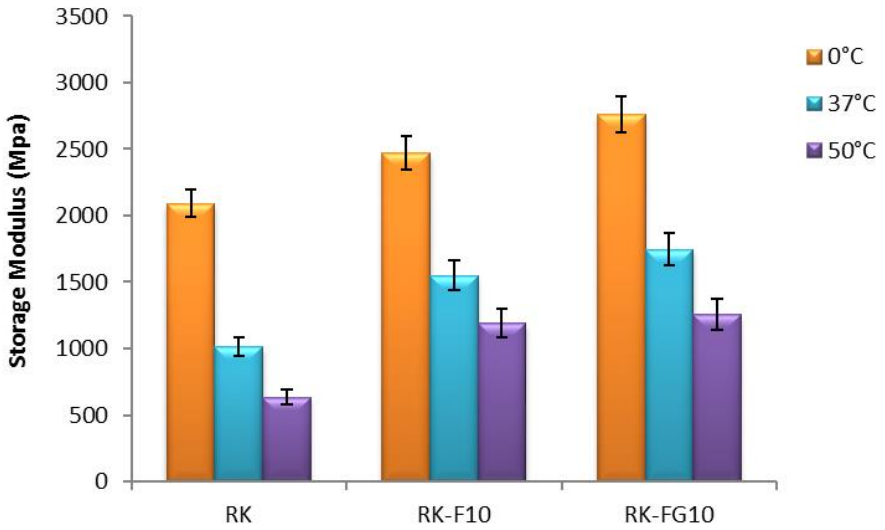

Figure 18: Storage moduli E' (MPa) at 0°C, 37°C and 50°C for RK, RK-F10 and RK-FG10.

5.2.4. RELEASE PROPERTIES

One of the main goal of this study, beside to improve the physical properties of the resins, is to obtain a resin able to release fluoride ions, in controlled and tuneable way according to the external environment to which the resin is exposed. The resin was suspended in physiological saline solution and the release of the active ingredient was monitored over time. A significant phenomenon was observed which constitutes a further advantage of the system: the anchorage of the active molecule to the inorganic lamellar compound allows slower release. This makes the system much more efficient. Figure 6 shows the release of fluoride ions (in ppm) at different initial concentrations from the RK-F0.7, RK-F5, RK-F10 and RK-F20 samples. A rapid

release at the beginning of the experiment, followed by a release linearly depending on the time (days) was observed. The first step is independent of the initial concentration, as expected for a glassy rigid polymeric matrix, in which the diffusive phenomena are only dependent on the frozen free volume. The second part of the release curve is linear with the release time. It inversely depends on concentration, in the sense that the higher the concentration the lower the released part of fluoride ions. This result reflects the strong influence of the morphology in the case of composites with lamellar clays. When the clay lamellae, where the fluoride ions are anchored, are delaminated and well dispersed in the resin, the fluoride ions, less shielded by the lamellae, are more available to be detouched from the clay and diffuse trough the resin. Increasing the clay concentration, big tactoids are formed and the fluoride ions are less available to be reached by the counter ions, detach and diffuse in the resin. Fluctuations between the different concentrations higher that 5%, are possibly due to morphology fluctuations during the composite preparation. This result indicates the possibility to have a tunable and controlled release of fluoride from RK resins. It is worth observing that the extrapolated release can occur up to one year, with a ppm concentration released each day that is far from the possible adverse fluoride effect (fig.19).

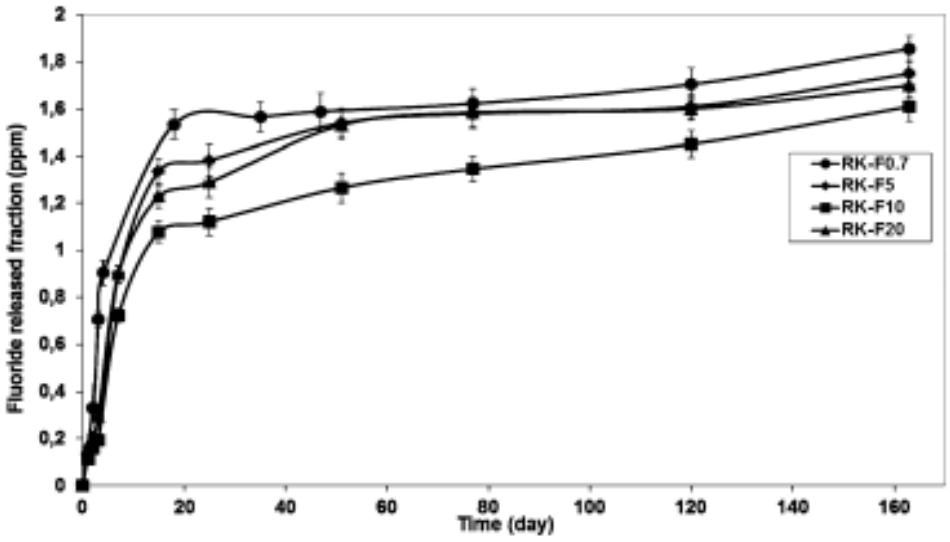

Figure 19: Release profiles of fluoride ions from RK-F0.7, RK-F5, RK-F10 and RK-F20 samples in physiological solution as a function of time (days).

In addition, cumulative fluoride release was evaluated for 28 days at 37°C in artificial saliva medium (SAGF) only for RK-F10 and RK-FG10 (Figure 7). The analyses were done every hour for the first 8 h, then daily for the first week and weekly until the end of the experiment. A time-dependent increase in the fluoride content was observed in SAGF for RK-F10 with respect to RK-FG10 at all time points tested (P <0.05). After 7 days of incubation, RK-FG10 released 2.723 ± 0.163 ppm of fluoride, an amount reached as early as 24 h. On the contrary, RK-F10 released 0.750 ± 0.053 ppm after 7 days with a daily mean increase of about 0.1 ppm, reaching a concentration of 1.667 ± 0.116 ppm at the end of the experiment. Nonsignificant differences of fluoride release were observed in cell culture medium (fig.20).

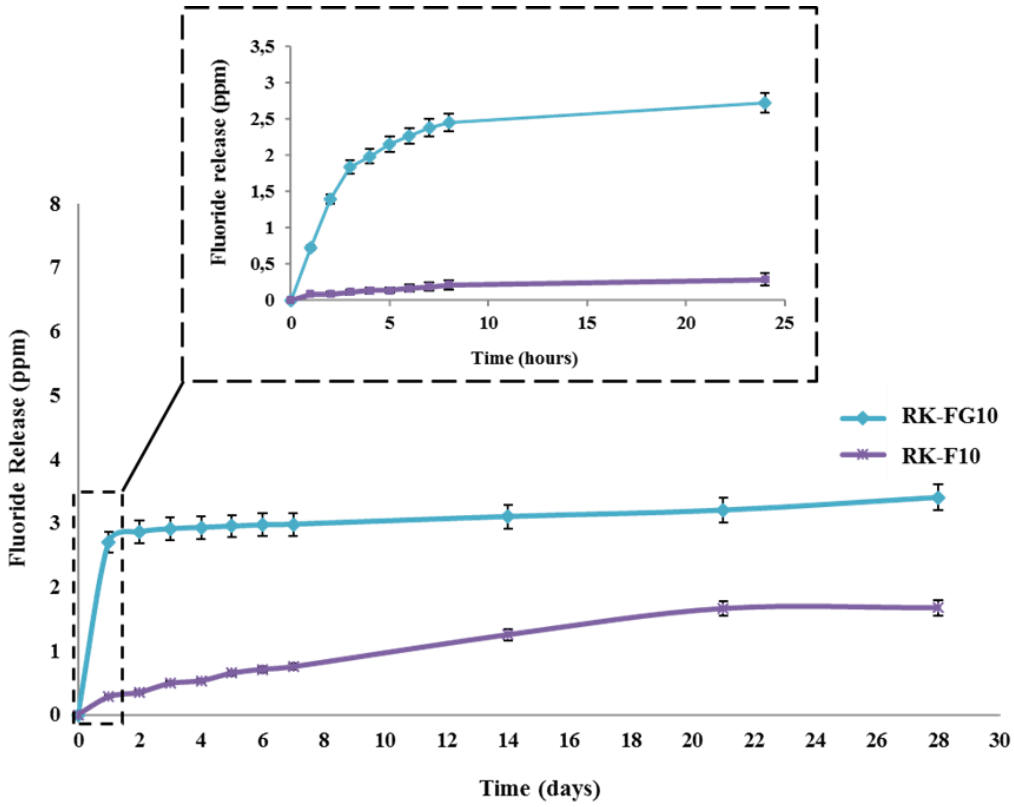

Figure 20: Cumulative release profiles of fluoride ions from RK-F10 and RK-FG10. Samples were put in mineral medium with composition similar to saliva (SAGF) for 28 days. The bars represent means ± standard deviation (n = 3). $P < 0.01$ for RK-F10 *versus* RK-FG10 at all-time points tested.

Our results demonstrated that, unlike conventional dental resins, fluoride-restorative materials modified with hydrotalcite present slow and constant fluoride-release kinetics.

5.3. DPSCs characterization

Human DPSCs can differentiate into various tissues, such as odontoblasts, adipocytes, chondrocytes, and osteoblasts [148, 160-162, 165-167]. In addition, hDPSCs are effective in mineralized tissue formation [168-170]. However, significant variability in growth patterns, CFU-F efficiency and stem cell immunophenotypic profiles have been recorded in hDPSC cultures established from different healthy donors of similar age and stage of third molar root development [171].

To identify the self-renewal potential of isolated cells of 10 donors, the ability of colony-forming unit-fibroblast (CFU-F) formation and their proliferation profiles were determined (figure 21a-b). CFU-F was normally observed within 1–2 weeks after cell seeding. A similar clonogenic cell population was observed for all donors. Figure 21a showed, just as an example, the number of CFU-F of 3 donors demonstrating that more than 80% of isolated cells were capable of forming colonies when seeded at low cell density.

The analysis of DPSCs proliferation demonstrated an increase time-dependent (figure 21b). The results showed that cells isolated from all donors exhibited a similar CFU-F and proliferation profile, therefore each of the following experiments was performed in triplicate on pooled cells.

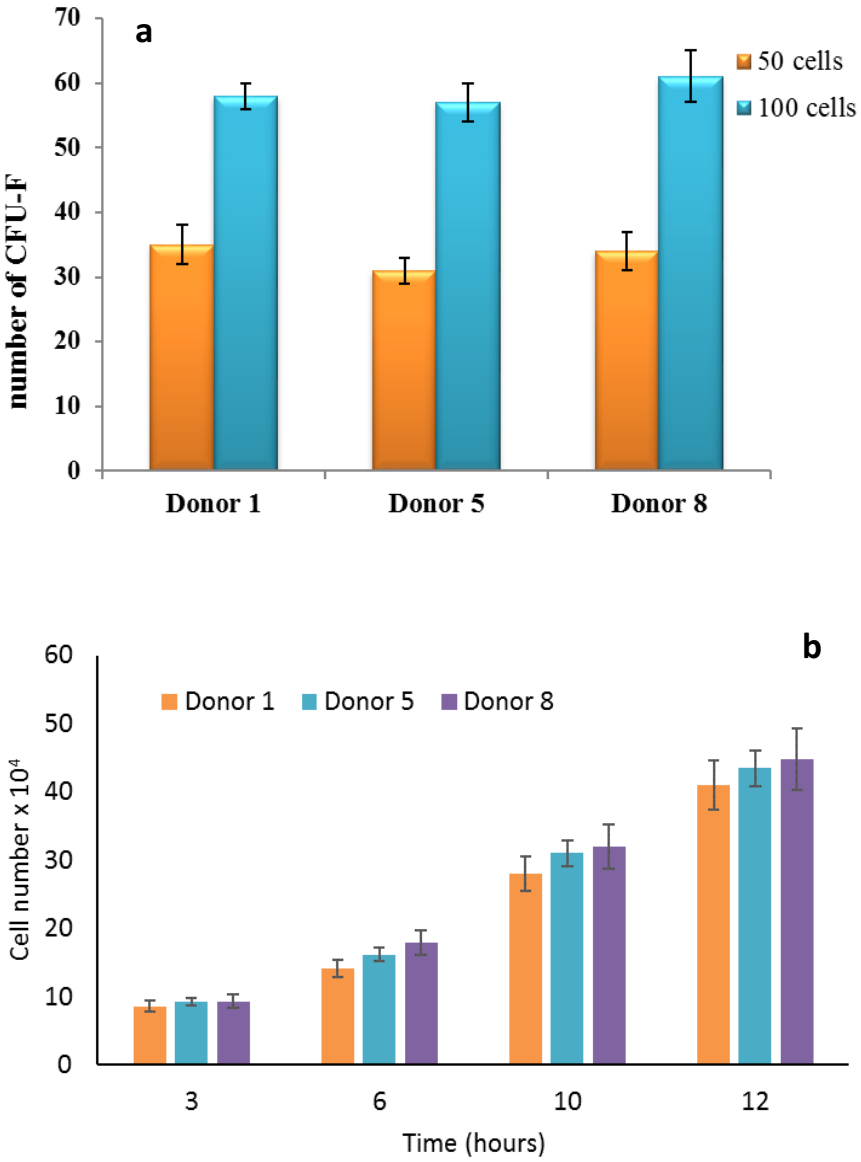

Figure 21: (a) The incidence of colony-forming cells from dental pulp cells of 3 selected donors at various plating densities. (b) Proliferation rate after 3, 6, 10 and 12 days of culture. The bars represent means ± SD for three experiments each performed in triplicate.

5.3.1. EFFECT OF FLUORIDE CONCENTRATION ON DPSCs

It has been hypothesized that the failure of dental restorations is dependent on the degree to which the pulpal cell populations can survive, as well as the ability of these cells to detect and respond to injury to initiate an appropriate repair response [172, 173]. It has been demonstrated that the level of fluoride release was in direct correlation with cytotoxic activity of fluoride-restorative materials on human DPSCs, while low levels of released fluoride correlated to low cytotoxic effect on human DPSCs [174]. To assess whether fluoride concentration affected the proliferation of DPSC, growth-arrested cells (in FBS-deprived basal medium for 24 h) were seeded in 0.2% FBS and then cell proliferation measured by PicoGreen dsDNA assay. Figure 22 demonstrated that cytotoxic concentration of fluoride was ranging between 3 and 5 ppm, a value did not present in the conditioned supernatant of fluoride release resins (RK-Fx and RK-FG10) for all the points evaluated either in physiological medium and SAGF.

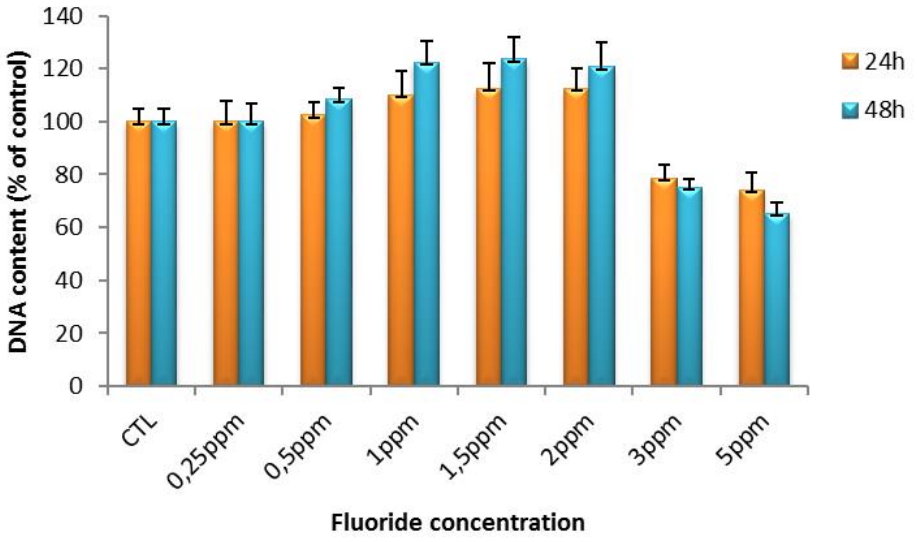

Figure 22. Effects of fluoride concentration on hDPSCs cell proliferation after 24 and 48 h. Cell cultured onto tissue culture polystyrene were used as control (CTL). The bars represent means ± SD for three experiments each performed in triplicate.

5.3.2. DPSCs OSTEOGENIC POTENTIAL

Magnetic-activated cell sorting (MACS) with antibodies against specific cell receptors can form a more standardized way of retrieving and culturing DPSCs. In particular, STRO-1 is a cell surface protein expressed by bone marrow stromal cells used to identify a population of stem cells able to differentiate into dental hard tissue-forming cells [175, 176]. These sorted stem cells (STRO-1^{+}) in high purity might provide a better cell source for therapeutic purposes than heterogeneous unsorted cells. Because of this, cells with pronounced expression of stem cell properties, including high growth potential, and high clonogenicity (CFU-F efficiency > 35%) were sorted by magnetic-activated cell sorting (MACS) with STRO-1 antibody. The amount of sorted stem cells (STRO-

1⁺ cells) ranged from 9 ± 2.5% to 14 ± 3.7%. Alkaline phosphatase activity (ALP), an early marker of odontogenic differentiation, was measured in both DPSCs and STRO-1⁺ cells cultured in osteogenic-induction medium. Cells cultured on growth medium (CTL) was used as control. Figure 23 showed that the ALP activity gradually increased for 28 days in cells grown in osteogenic-induction medium respect to cells cultured onto growth medium ($P < 0.01$). Moreover, ALP activity of STRO-1⁺ cells increased ($P < 0.05$) compared to that of DPSCs.

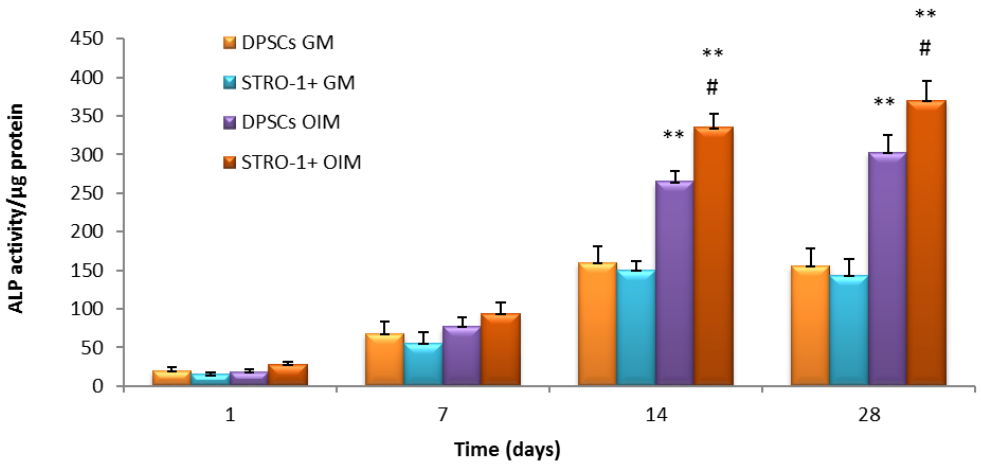

Figure 23: ALP activity of DPSCs and STRO-1⁺ cells culuterd in growth (GM) and osteogenic-induction medium (OIM). The bars represent means ± SD for three experiments each performed in triplicate. Statistically significant difference ** $P < 0.01$ *versus* DPSCs GM and STRO-1⁺ GM; # $P < 0.05$ *versus* DPSCs OIM and STRO-1⁺ OIM.

5.3.3. EFFECTS OF FLUORIDE ON STRO-1$^+$ CELL MIGRATION

It is well known that fluoride can affect sperm chemotaxis and the human osteoblastic cell migratory response to chemotactic factors in a dose-dependent manner [136, 177]. However, no study has investigated the role played by fluoride on DPSC ability to migrate in response to different stimuli or the influence of the fluoride level on cell migration. The migratory response of the STRO-1$^+$ cells cultured for 7 days on RK, RK-F10, and RK-FG10 materials (STRO-1$^+$ cells/RK, STRO-1$^+$ cells/RK-F10, and STRO-1$^+$ cells/RK-FG10, respectively) was examined using a modified Boyden chamber in the presence of different chemoattractants (TGF-β1 or SDF-1). STRO-1$^+$ cells/RK-F10 showed a significant ability ($P < 0.05$ vs. CTL, RK, and RK-FG10) to respond to cytokines, while a modest cell migration in the presence of both chemoattractants was exhibited by STRO-1$^+$ cells/RK-FG10 (figure 24).

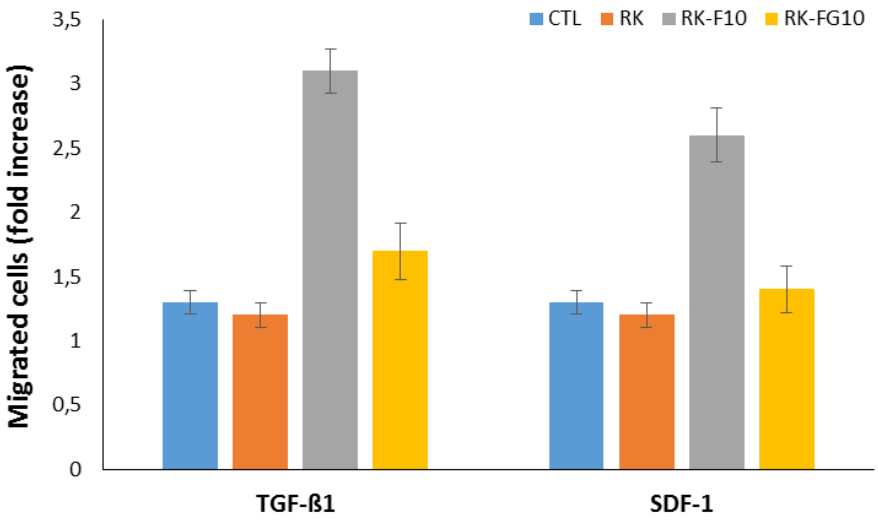

Figure 24: Chemotactic migration of STRO-1$^+$ cells in presence of TGF-β1, and SDF-1. Cell migration assays were evaluated using a modified Boyden chamber. Cells were cultured on RK, RK-F10 and RK-FG10 materials for 7 days before the migration assay was performed. The bars represent means ± SD for three experiments each performed in triplicate. Statistically significant difference * $P < 0.05$ *versus* CTL, RK, and RK-FG10.

The results demonstrated that long-term exposure of STRO-1$^+$ cells to a continuous release of a low amount of fluoride from RK-F10 resin increased their migratory response to TGF-β1 and SDF-1, both important promoters of pulp stem cell recruitment. On the contrary, the initial burst of fluoride from RK-FG10 resin (RK filled with fluoride-glass filler) led to a higher initial fluoride delivery which, however, reduced the effective lifetime of the delivery and the biological effects of fluoride on cell chemotaxis. It is possible that the effects of a low concentration of fluoride on modulating cell migration might be related, at least in part, to its well-known ability to activate G protein, initiating signaling cascades that regulate the migration of different cell types [137, 178].

5.3.4. EFFECTS OF FLUORIDE-RELEASING MATERIALS ON THE DIFFERENTIATION OF STRO-1^+ CELLS

Functional differentiation of odontoblasts, the second step in dentinogenesis, requires unique sets of genes to be turned on and off in a growth and differentiation-specific manner [179]. To our knowledge, few studies have investigated the effect of long- term fluoride exposure on DPSC differentiation. As reportedby Thaweboon et al. [8], fluoride at low concentrations can stimulate proliferation and differentiation of DPSCs, whereas an inhibitory effect can be observed at higher concentrations. In addition, Wurtz and colleagues [14] demonstrated that the gene expression profile of the odontoblast-like cell line MO6-G3 changed in response to a low fluoride concentration, without inducing cell stress or apoptosis.

To analyze the effect of fluoride release kinetics from RK-F10 and RK-FG10 on the differentiation of STRO-1^+ cells, the expression of several genes coding proteins that constitute dentin, the major component of dental mineralized tissue was followed: osteocalcin (OCN), dentin matrix protein-1 (DMP-1), dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE). In particular, OCN, DSPP, and DMP-1 were chosen as late markers for odontogenesis, while MEPE was chosen as early marker [102, 180-183]. During dentinogenesis, OCN can be found within intracellular vesicular structures in the odontoblast processes, with a tightly regulation of its temporal and spatial expression; DMP1 is expressed in all mineralized tissues, and its expression is directly linked to amelogenesis, cementogenesis, odontogenesis, chondrogenesis, and osteogenesis during both embryonic and post-natal development, having an important role in matrix mineralization through the regulation of crystal size and morphology. Similarly, to OCN and DMP1, also DSPP was chosen as late marker for

odontogenesis. DSPP is a highly phosphorylated non-collagenous protein that is cleaved immediately after secretion into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), two tooth-specific proteins [182].

While all of these molecules enhance differentiation and mineralization, an early differentiation marker is MEPE, also called osteoblast/osteocyte factor 45, a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family. Even if its exact role as a regulator of mineralization and the mechanisms underlying this function are still controversial, its expression is only observed in immature odontoblasts, and becomes downregulated upon odontoblastic differentiation [180, 184].

The expression of *ocn, dspp, dmp1, mepe* was examined using real-time PCR on STRO-1+ cells over a period of 28 days of culture on RK-F10 and RK-FG10. Cells cultured on polystyrene (CTL) and on fluoride-free resin (RK) were used as controls. Quantitative PCR showed that *mepe* messenger RNA (mRNA) was significantly higher in STRO-1^+ cells/RK-F10 ($P < 0.001$ *vs*. CTL, RK, and RK-FG10) as early as 3 days of culture and was downregulated for the rest of the culture period (figure 25). The expression of later marker genes (*ocn, dspp, dmp1*) of odontoblast differentiation was upregulated throughout the experiment in STRO-1^+ cells/RK-F10 compared with CTL, RK, and RK-FG10. Although a significant *ocn* and *dmp1* gene expression was observed in STRO-1^+ cells/RK-FG10 ($P < 0.01$ *vs*. CTL and RK), this resin was not able to modulate the expression of all chosen odontoblast markers throughout the experiment. In fact, *ocn, dspp, dmp1*, and *mepe* mRNA levels remained almost constant over the culture period in STRO-1^+ cells/RK-FG10.

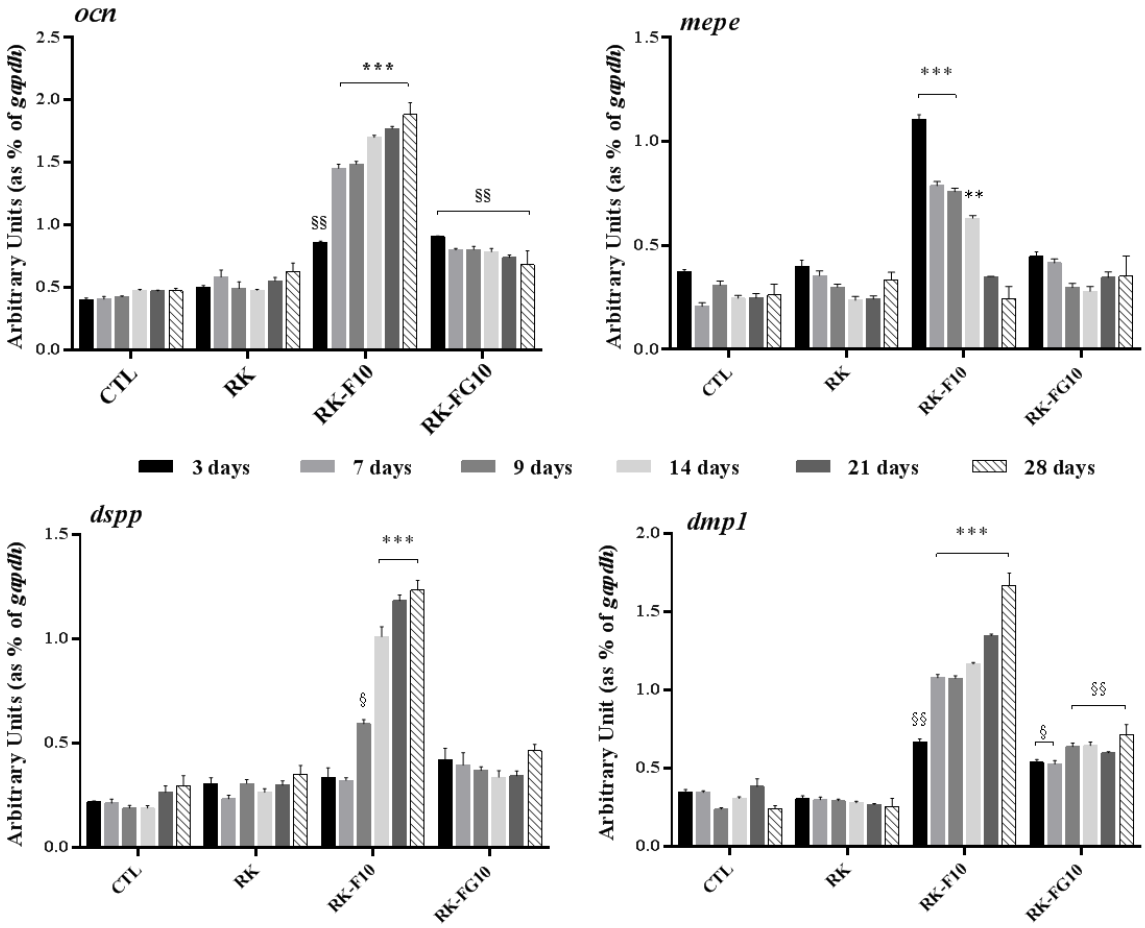

Figure 25: Quantitative RT-PCR analysis of *ocn*, *mepe*, *dspp*, and *dmp1* in STRO-1$^+$ cells cultured in presence of RK-F10 or RK-FG10 for 3, 7, 9, 14, 21 and 28 days. Cell cultured on tissue culture polystyrene (CTL) or on RK were used as controls. The target gene expression was normalized to the housekeeping gene *gapdh*. Relative differences in PCR results were calculated using the comparative cycle threshold (CT) method. The bars represent means ± SD for three experiments each performed in triplicate. Statistically significant difference ** $P < 0.01$ *versus* CTL, RK, and RK-FG10; *** $P < 0.001$ *versus* CTL, RK, and RK-FG10; § $P < 0.05$ *versus* CTL, and RK; §§ $P < 0.01$ *versus* CTL, and RK.

6. Conclusions and Future directions

In this thesis the formulation, preparation and characterization of novel visible-light cured composites based on photo-activated Bis-GMA/TEGDMA matrix, containing a filler based on an hydrotalcite-like compound intercalated with fluoride ions was discussed. In contrast to the conventional glass-ionomers, RK-Fx showed improved physical properties and no initial toxic fluoride 'burst' effect and levels of fluoride release remain relatively constant over time.

In addition, this thesis demonstrated that the differentiation and chemotaxis capacity of hDPSCs were modulated by long-term controlled delivery of micromolar amounts of fluoride using a modified-hydrotalcite restorative dental resin (RK-F10). The use of fluoride as a supplementary material to improve the dental materials already existing and widely used seems very promising and has led to the commercialization of F-RMs as remineralizing agents in the treatment of "caries-affected" dentin. Since there is no general consensus on the local threshold dose of fluoride or on the duration of exposure to fluoride necessary to elicit a restorative effect, the commercially available F-RMs have dramatically different fluoride-releasing profiles. Recently, several studies showed a definite correlation between fluoride release from different F-RMs and increased mortality of DPSCs. Kanjevac et al. [174] evaluated the potential cytotoxic effects of commercial biomaterials on hDPSCs and showed that F-RMs that released fluoride in high quantities were more toxic to hDPSCs than materials releasing low levels of fluoride. However, there is currently no direct proof that long-term release kinetics of nontoxic amounts of fluoride from F-RMs can affect the migration, proliferation, and differentiation of DPSCs.The involvement of fluoride at low concentrations (0.05–0.4 ppm) [185] seems to be necessary for enamel mineralization, but it is of paramount interest also to know whether the same concentrations can

modulate DPSC functions. Indeed, DPSCs play a fundamental role in the repair mechanism against dental damage by migrating toward the injury site and, once in place, differentiating into odontoblast- like cells and forming reparative dentin [186].

Moreover, the ability of LDH to exchange anions in acidic environment could be useful to increase the release of fluoride in case of secondary caries. In this case increased levels of fluoride might have potential antibacterial action.

In conclusion, the results reported herein highlight the importance of taking into account the fluoride release kinetics in addition to the fluoride concentration when designing new fluoride-restorative materials.

Indeed, new tissue formation guarantees restoration duration and stability, due to the reduced bacterial infiltration and secondary caries development.

The next step will be *in vivo* analysis to confirm the effect of fluoride relase kinetic in tooth environment.

The studies discussed in this thesis were published

as: *Journal of dentistry 2014; 42(1):60–67*

Journal of Dental Research 2015; 94(8):1099-105

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