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BIOENGINEERED TOOTH EMULATION SYSTEMS FOR REGENERATIVE AND PHARMACOLOGICAL PURPOSES

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Abstract

Genetic conditions, traumatic injuries, carious lesions and periodontal diseases are all responsible for dental pathologies. The current clinical approaches are based on the substitution of damaged dental tissues with inert materials, which, however, do not ensure full physiological recovery of the teeth. Different populations of dental mesenchymal stem cells have been isolated from dental tissues and several attempts have already been made at using these stem cells for the regeneration of human dental tissues. Despite encouraging progresses, dental regenerative therapies are very far from any clinical applications. This is tightly connected with the absence of proper platforms that would model and faithfully mimic human dental tissues in their complexity. Therefore, in the last decades, many efforts have been dedicated for the development of innovative systems capable of emulating human tooth physiology *in vitro*. This review focuses on the use of *in vitro* culture systems, such as bioreactors and "organ-on-a-chip" microfluidic devices, for the modelling of human dental tissues and their potential use for dental regeneration and drug testing.

Keywords: Tooth, dental pulp stem cells, periodontal ligament stem cells, bioreactors, microfluidics, organ-on-a-chip, stem cells, drug discovery, regeneration.

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Introduction

Tooth pathologies affect the totality of the population, with a significant burden for global healthcare systems. Genetic conditions, traumatic injuries, caries and periodontal diseases can lead to tooth pathology and loss (Caton *et al.*, 2011; Pagella *et al.*, 2015). Clinical approaches consist mostly in the substitution of the damaged dental tissues with specific materials, resulting however in the loss of the vital components of the teeth and in a significant impairment of their functionality (Orsini *et al.*, 2018a). Devitalised teeth show many undesirable characteristics, including strength reduction, increased fragility and predisposition to postoperative fracture (Adell *et al.*, 1990; Caton *et al.*, 2011; Esposito and Worthington, 2013; Fron Chabouis *et al.*, 2013; Pagella *et al.*, 2015). In case of tooth loss, implants are commonly used. While widely employed, implants are prone to infections and do not ensure the physiological functions of teeth (Callan, 2007; Caton *et al.*, 2011; Orsini *et al.*, 2018a; Pagella *et al.*, 2015). To respond to these great unmet clinical needs, increasing attention was dedicated in the last years to the development of culture systems that emulate the physiology of human dental tissues and allows the study of their response to environmental and pharmacological stimuli as well as the generation of fully functional dental tissues.

Tooth anatomy, development and pathology

Teeth are composed of a unique combination of hard and soft tissues (Nanci, 2013; Pagella *et al.*, 2020c). The enamel is the hardest tissue of the human body and covers the crown of the tooth. The enamel is supported by the dentine, a second highly mineralised tissue. The central portion of the tooth is occupied by

the dental pulp, a connective tissue that conveys innervation and vascularisation. Nerve fibres from the trigeminal ganglion enter the dental pulp from the apical foramen and extend until within the dentine, conducting pain and sensitivity. The dental pulp is supported by a rich vascularisation, which guarantees trophic support and tooth survival and regeneration. The tooth is anchored to the surrounding alveolar bone through the periodontium, which absorbs the various shocks associated with mastication and provides tooth stability by continuously remodelling its extracellular matrix, the periodontal ligament (Fig. 1**a**) (Nanci, 2013; Pagella *et al.*, 2020c). Similarly to the dental pulp, the periodontal ligament is supported by a rich vascular plexus and a rich neuronal network.

The development of the tooth results from sequential and reciprocal interactions between cells of the oral epithelium and the cranial neural-crest-derived mesenchyme (Kollar, 1986). Oral epithelial cells generate ameloblasts that produce the enamel and then drive the development of the dental roots (Diekwisch, 2001; Mitsiadis and Graf, 2009; Nanci, 2013). Dental mesenchymal cells generate odontoblasts, the cells responsible for dentine production, as well as the dental pulp and the periodontium (Mitsiadis and Graf, 2009; Nanci, 2013). In pathological conditions, such as mild carious lesions, odontoblast activity is stimulated to elaborate a reactionary dentine (Smith *et al.*, 1995). Dental traumas involving violent stresses (*i.e.* cavity preparations) lead to odontoblast death and newly formed odontoblast-like cells generate reparative dentine (Fig. 1**b**) (Goldberg *et al.*, 2011). In case of an extensive injury or infection, the reparative and regenerative responses of the dental pulp are not sufficient and the dental pulp undergoes necrosis (Orsini *et al.*, 2018b). The periodontium is continuously exposed to mechanical, chemical and bacterial insults. Periodontal injuries and infections are often not compensated by sufficient regenerative responses and, thus, represent the most frequent cause of tooth loss (Papapanou, 1996).

Both the dental pulp and the periodontium contain mesenchymal stem cells (MSCs), named dental pulp stem cells (DPSCs) and periodontal stem cells (PDLSCs), respectively (Fig. 1**a**) (Gronthos *et al.*, 2000; Roguljic *et al.*, 2013). DPSCs and PDSCs are multipotent and respond to cellular, chemical and physical stimuli to ensure homeostasis and regeneration of dental tissues. In the last decades, isolated DPSCs and PDSCs have been the subject of intense investigation as possible tools for the

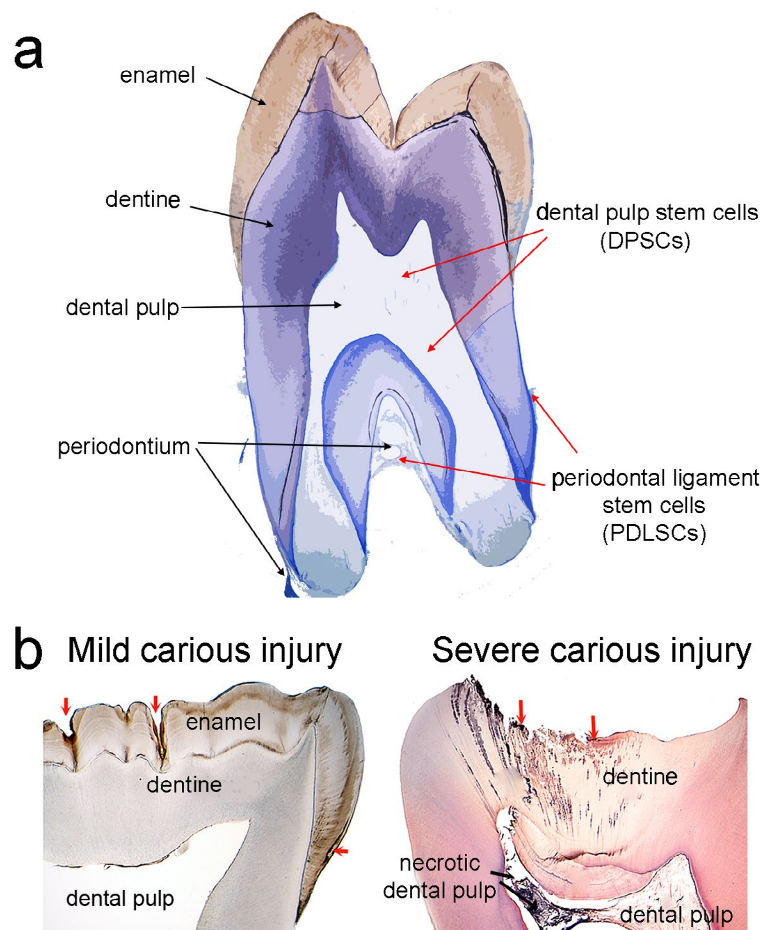

Fig. 1. Tooth anatomy and histology in physiological and pathological conditions. (**a**) Schematic representation of a human premolar. (**b**) Histological sections of human carious teeth. Left: ground section, unstained, showing mild carious injuries (red arrows) affecting only enamel. Right: decalcified section, haematoxylin and eosin, showing a severe carious injury (red arrows) with bacterial invasion (asterisks: bacterial front within dentine). Adapted from Orsini *et al.* (2018b).

regeneration of both dental and non-dental tissues (Orsini *et al.*, 2018a). *In vivo* studies aiming at the regeneration of dental pulp and periodontium are promising but they have not managed yet to recreate fully functional tissues (Chen *et al.*, 2020; Xu *et al.*, 2019; Xuan *et al.*, 2018). This can be attributed to the limited information that is currently available on the function and properties of human MSCs in dental pulp and periodontium *in vivo* and their interaction with their microenvironment. Stem cell behaviour is indeed regulated by molecular cues produced in their microenvironment (also called the stem cell niche) by stromal cells, neurons, vascular-related cells and immune cells as well as by physical factors such as shear stress, stiffness and topography (Lane *et al.*, 2014; Pagella *et al.*, 2021). Despite their biological and clinical relevance and although the very recent publication of the single-cell atlas of human teeth (Pagella *et al.*, 2021), a thorough understanding of the interactions between dental stem cells and their microenvironment is still lacking. This is tightly connected with the absence of proper platforms that would model human dental tissues in all their complexity.

Platforms for studying human dental tissues *in vitro*

Many studies have been performed in classical bidimensional (2D) culture systems and these have been pivotal to characterise the basic properties of the various dental stem cell populations (Mitsiadis *et al.*, 2015). However, the translation of these results to the clinical practice has been very limited, highlighting the need for more accurate emulation systems (Skardal *et al.*, 2016). Indeed, these 2D culture systems lack most of the features that characterise dental tissues *in vivo*, such as interactions between the cells and the extracellular environment as well as with other cell types (Pampaloni *et al.*, 2007). Three-dimensional (3D) culture systems have been developed to overcome some of these limitations. 3D structures such as spheroids and organoids allow complex cell-cell interactions, creation of gradients of oxygen, circulation of nutrients and soluble signals, which lead to the generation of tissue-specific heterogeneous cell types (Yin *et al.*, 2016). Spheroids have been generated from both dental epithelial and mesenchymal stem cells (Berahim *et al.*, 2011; Natsiou *et al.*, 2017; Stevens *et al.*, 2008) and have displayed the expression of tissue-specific differentiation gradients when compared to 2D culture systems (Berahim *et al.*, 2011). 2D and 3D culture systems have been most recently complemented by increasingly more complex systems that allow for the study of dental stem cells in the presence of the other cell types that compose their niches as well as their responses to physical and chemical stimuli. The following sections focus on the use of these systems, including bioreactors and microfluidic "organ-on-a-chip" devices, for the emulation and regeneration of human dental tissues and the study of their response to pharmacological treatments (Table 1, 2).

Various models of dental tissue-specific bioreactors

Bioreactors have been adopted as a strategy to create more accurate and complex biological models that better mimic pathophysiological conditions, to recreate brand new dental tissues or test novel pharmaceutical products. Such devices are meant to overcome static culture limitations, enhance oxygenation and nourishment of the constructs

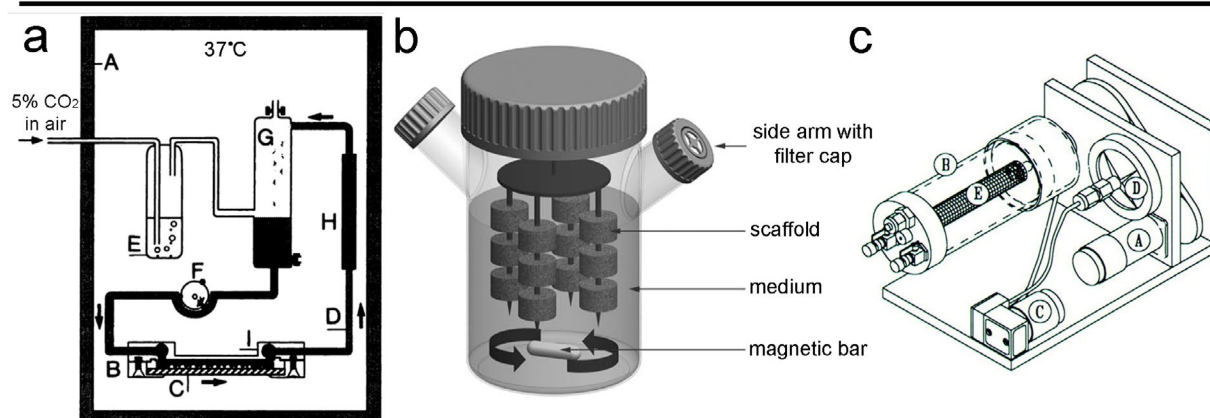

Fig. 2. Overview of perfusion and shear stress bioreactors used for the study and emulation of human dental tissues. (**a**) Adapted from Kraft *et al.* (2010). (**A**) Incubator; (**B**) parallel plate flow chamber; (**C**) glass slide with cell monolayer; (**D**) fluid culture medium; (**E**) H_20; (**F**) revolving pump; (**G**) gas phase of 5 % CO_2 in air; (**H**) flow probe; (**I**) polycarbonate plate with two slits through which medium enters and exits the channel. (**b**) Adapted from Woloszyk *et al.* (2014). (**c**) Adapted from Hammond and Hammond (2001). (**A**) A 24 V direct-current motor drives a belt that rotates the (**B**) cylindrical culture vessel along its horizontal axis. (**C**) An air pump draws incubator air through a (**D**) 0.22 µm filter and discharges it through a rotating coupling on the shaft that carriers the vessel. (**E**) The oxygenator is wrapped around the centre post.

and, depending on their design, they are able to provide different ranges and frequencies of mechanical stimulation (Wendt *et al.*, 2009). Physical conditioning parameters are chosen according to the cues experienced by the tissues in homeostasis, developmental and pathological stages. Dynamic culture systems are in particular crucial for tissue engineering advancements and their translation to clinical studies.

Perfusion and shear stress bioreactors

Perfusion of the constructs enhances transport of oxygen and nutrients that thus are able to reach the inner parts of the scaffold. At the same time, perfusion can be used to induced shear stress applied to the cells. One of the first studies involving this type of stimulation for dental application adopted a parallel-plate chamber and a fluidic circuit to convey a pulsatile flow to a DPSC monolayer (Kraft *et al.*, 2010). Such system consisted of a polycarbonate plate culture chamber where medium flow is driven by a peristaltic pump drawing from an air-filtered reservoir (Fig. 2**a**). This configuration enables to finely tune the shear stress applied to the cells by varying the fluid flowrate. Furthermore, the roller pump is responsible for the pulsatile motion. The drawbacks are related to the 2D culture limited ability to produce functional tissues for regeneration or complex models. It is possible to include a scaffold in the chamber by changing its design and assembling procedures. In this instance, in case of direct perfusion, finding a proper flowrate threshold is mandatory since the medium is forced to flow through the scaffold pores and high values of shear stress could impair cell adhesion and viability. Perfusion and shear stress can also be conveyed by means of batch systems such as Spinner Flask bioreactors or Rotating Wall Vessel bioreactors (RWV), which are the most versatile in terms of cell types and scaffold to be employed. Spinner Flask devices are simple and easy to use: a large chamber is filled with culture medium and seeded scaffolds are placed on specific holders. Air

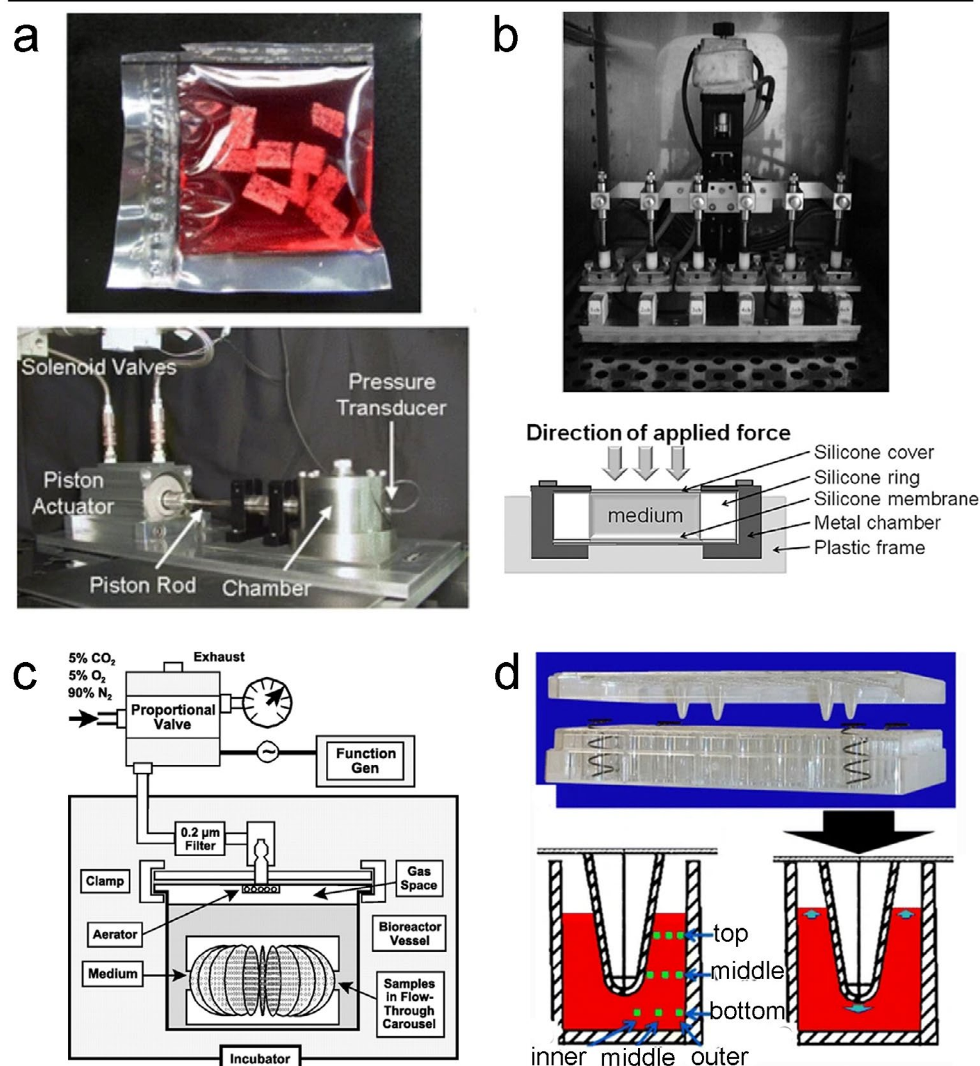

Fig. 3. Overview of hydrostatic pressure and compression bioreactors used for the study and emulation of human dental tissues. (**a**) Adapted from Reza and Nicoll (2008). (**b**) Adapted from Miyashita *et al.* (2017). (**c**) Adapted from Wenger *et al.* (2011). (**d**) Adapted from Ji *et al.* (2014).

filters are used to guarantee both oxygenation and sterility, while medium motion and shear stress on the surface of the constructs are provided by stirring bars (Fig. 2**b**). This dynamic culture method has already been adopted with human DPSCs (hDPSCs) seeded on silk fibroin scaffolds to investigate their differentiation behaviour (Woloszyk *et al.*, 2014). As for the RWV system, it has been previously used to culture enamel organ epithelium cells on dextran microbeads (Li *et al.*, 2012a). It consists of a cylindrical vessel and an internal coaxial oxygenator while cells are seeded on microcarriers or scaffolds (either free to move or placed on holders). The motion of the medium is provided by slow rotation of the vessel and the modulus of the stimulation is related to the revolutions per minute (Fig. 2**c**). In general terms, batch bioreactors employ large amount of culture medium and costs of reagents must be considered if expensive chemicals, such as growth factors, are needed. Furthermore, mass transport towards the bulk of the seeded material is limited, particularly after long culture periods because cells proliferation and extracellular matrix (ECM) production on the surface prevent deeper cells from receiving nutrients. Cross contamination between samples cannot be avoided with such methods and control of the stimulation is also limited, except for the RWV system whose rotation causes laminar flow and nearly homogenous shear fields. Other disadvantages are related to the need to manually replace exhausted medium and to the bumping between carriers or scaffold and the walls that can impair cells functionality. A custom-made apparatus has been previously used to provide titanium implants with tissue engineered periodontal ligament (PDL) (Gault *et al.*, 2010). Briefly, PDL cells cushions were cultured on hydroxyapatite (HAP)-coated titanium pins, incubated in vessels and tested for bone integration after implantation. In this case, perfusion of the cellular cushion was obtained *via* the medium flow occurring through the small gap between the pin and the walls of the vessel.

Hydrostatic pressure and compression bioreactors

Hydrostatic pressure is crucial for all tissues that transduce forces, such as those filled with fluids or highly hydrated, as their loading results in

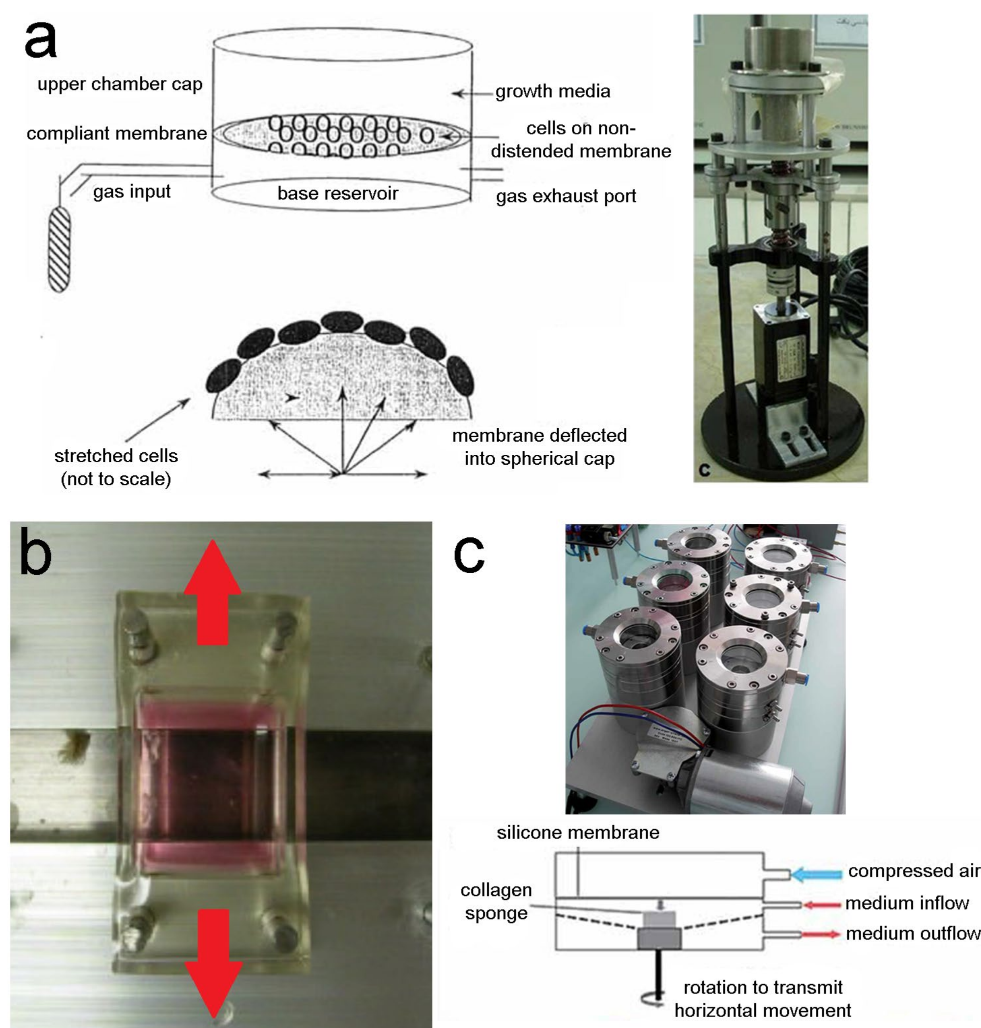

Fig. 4. **Overview of strain and combined bioreactors**. (**a**) Adapted from Howard *et al.* (1998). (**b**) Adapted from Hata *et al.* (2013). (**c**) Adapted from Mathes *et al.* (2010).

interstitial pressure modification (Reinwald and El Haj, 2018). A typical hydrostatic pressure bioreactor includes a chamber and a piston, which regulates its pressure. The chamber contains scaffolds and medium while pressure is conveyed equally to all surfaces in contact with the liquid phase. A similar system has been used with hDPSCs cultured on glass substrates: seeded glass coverslips were put in a plastic bag filled with medium and placed in a pressure chamber containing water (Fig. 3**a**). This "sack technique" guarantees sterility and allows the bioreactor chamber to be instantly reusable without the need for complex assembling procedures (Yu *et al.*, 2009). A more sophisticated apparatus was designed to culture hDPSCs adhered to micro-patterned membranes and stimulate them with cyclic pressure strain (Fig. 3**b**). More specifically, the system comprised six metal chambers, each housing a sample of seeded membrane on the bottom. Silicone rings and covers ensured watertightness and sterility while pressure was applied through external pistons-actuator elements that pushed on deformable silicone covers. This pressure was hydraulically conveyed to cells thanks to the medium. This bioreactor is characterised by great versatility and the presence of multiple chambers enables to use independent samples without the risk of cross contamination, while facilitating the investigation of biological events at different time points (Miyashita *et al.*, 2017). In another study, a high throughput system hydrostatic pressure was designed by hosting previously seeded coverslips in a polycarbonate "carousel" holder with 18 radial slots (Wenger *et al.*, 2011). The holder was placed in a vessel containing medium and a controlled amount of gas (Fig. 3**c**). By changing the amount of gas, different magnitudes of pressure can be applied cyclically, while the composition of the gaseous phase enables to have custom concentrations of oxygen and carbon dioxide regardless of the concentrations inside the incubator. The filtered injection of gases is performed by a pumping system placed outside of the incubator due to its hindrance.

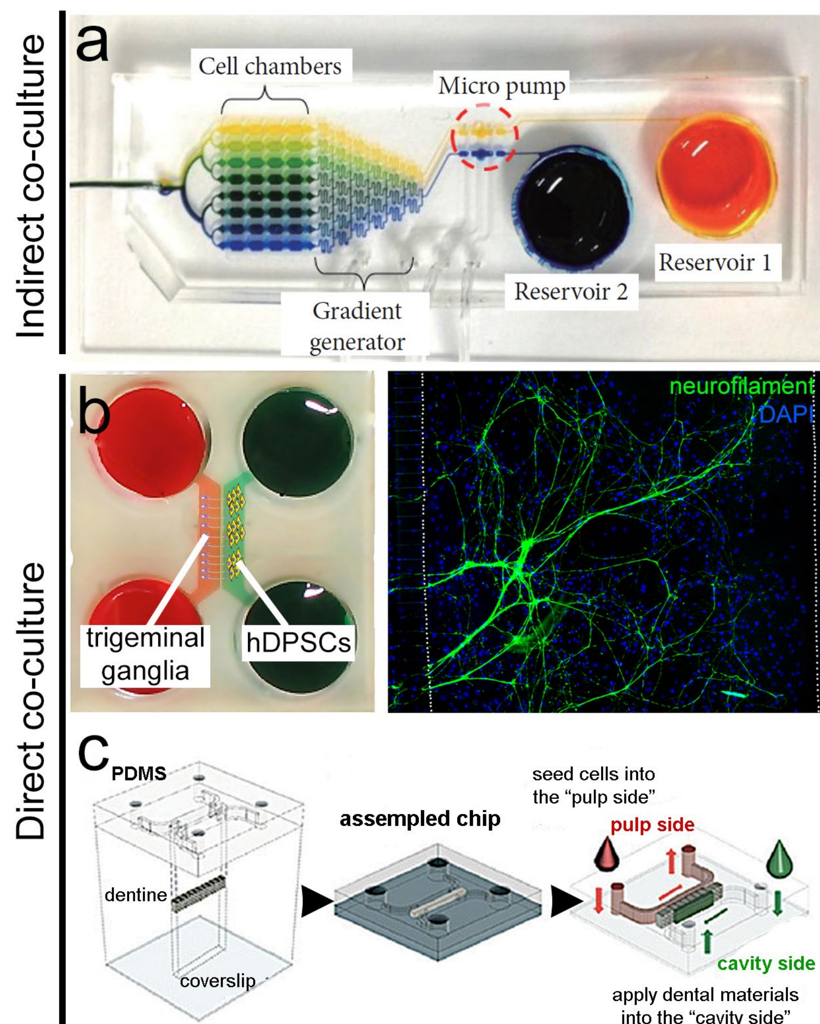

Fig. 5. Overview of microfluidic devices used for the study of human dental tissues. (**a**) Microfluidic device for the study of the effects of different concentrations of factors secreted by human gingival fibroblasts and human periodontal ligament stem cells on human dental pulp populations. Adapted from Kang *et al.* (2016). (**b**) Microfluidic device used for the co-culture of trigeminal ganglia or neurons and hDPSCs. Adapted from Pagella *et al.* (2020b). (**c**) Microfluidic device used for the co-culture of hDPSCs and dentine fragments. Adapted from Franca *et al.* (2020).

Table 1. Bioreactors for mechanical conditioning in dental tissues engineering.

In vitro model	Mechanical stimulation	Scaffold	Culture type	Advantages	Disadvantages	Application	Reference
Perfusion [pulsating fluid flow (PFF)]	Pulsatile shear	Glass coverslip	2D	• Finely tuneable shear	• No tissue complexity	DPSC monolayer dynamic culture	Kraft *et al.*, 2010
Spinner Flask	Shear	Silk fibroin	3D	• Versatility • Easy to use	• Wide stimulation range • Batch system • Bulk mass transport	Investigation of hDPSC differentiation	Woloszyk *et al.*, 2014
RWV	Shear	Dextran micro-beads	2D	• Versatility • Homogeneous and tuneable shear fields	• Batch system • Bulk mass transport • Possible scaffold damage	Investigation of enamel organ epithelial cells cells differentiation into ameloblasts	Li *et al.*, 2012
Perfusion	Shear	Titanium pins	2D	• Tuneable flow • Effective mass transport • Functional tissues	• Flowrate maximum threshold	Tissue engineered periodontal ligament	Gault *et al.*, 2010
Pressurised chamber	Hydrostatic pressure (HSP)	Glass coverslip	2D	• Tuneable stimulation • Easy to use • Less chance of contamination (bag technique) • Instantly reusable (bag technique)	• Batch culture	DPSC dynamic culture on glass substrates	Mhatre *et al.*, 2012
Piston-actuator	HSP	Patterned silicone membrane	2D	• Tuneable stimulation • Low risk of cross-contamination	• No tissue complexity	Study of DPSC differentiation behaviour depending on stimulation and patterned surfaces	Miyashita *et al.*, 2017
"Carousel" system	HSP	Glass coverslip	2D	• High throughput • Custom gas concentration • Easy assembling and sample handling • Tuneable stimulation	• No tissue complexity • Batch culture	hPDLSCs dynamic culture	Wenger *et al.*, 2020
Microplate system	Compression	Agarose gel	3D	• Inexpensiveness • Reduced hindrance • Ease to assemble	• Position-dependent stimulation	Investigation of loading effects on hDPSC osteogenesis	Sun *et al.*, 2014
Pneumatic or piston actuation	Isotropic strain	Silicone membrane	2D	• Homogeneous stimulation • Simple system	• No tissue complexity	hPDLF or hDPSC dynamic culture	Howard *et al.*, 1998; Tabatabaei *et al.*, 2014
Deformable chamber	Uniaxial strain	Silicone membrane	2D	• Easy optical evaluation • High reproducibility	• No tissue complexity	Investigation of DPSC osteogenesis and adipogenesis	Hata *et al.*, 2013
Moveable anchors	Uniaxial strain	Woven silk fibroin	3D	• Tuneable stress • Functional tissue	• Unpredictable mass transport • Bulk mass transport	Tissue engineered alveolar bone	Han *et al.*, 2010
Combined system	Compression + shear	Collagen sponge	3D	• Multiple stimulation • Physiological mechanical model	• Bulk mass transport	Engineered soft oral tissues	Mathes *et al.*, 2010

Table 2. Microfluidic culture systems used for dental tissues engineering.

In vitro model	Features	Application	References
Indirect culture system	High throughput Versatility Low sample consumption	Investigation of hGF hPDLSCs secreted factor influence on (stem cells from human exfoliated deciduous teeth (SHED) population	Kang *et al.*, 2016
Direct culture system	Physiological microenvironment model Versatility	Tooth innervation studies during developmental stages	Pagella *et al.*, 2014; 2020a; 2020b
Dentine-resin interface	Physiological microenvironment model Dentine fragment Live imaging	Model of stem cells from the apical papilla (SCAP)-derived odontoblast and restorative resin interaction	Franca *et al.*, 2020

Another advantage of such design is the large number of samples stimulated at the same time and the easy assembling procedure (Wenger *et al.*, 2011).

Compression stimulus is another valid choice for load-bearing tissues and it is conveyed by directly deforming the seeded surface or scaffold. The alveolar bone is continuously loaded during chewing movement; hence, proper magnitudes of compression are considered to favour differentiation toward osteogenesis. A bioreactor was designed to mimic forces applied to alveolar bone cells during chewing motion (Ji *et al.*, 2014). Such bioreactor can be assembled starting from common lab equipment. Four wells of a microplate constitute the culture chambers containing a cell-laden agarose gel. Pressure is exerted on each of them by the bottom part of Eppendorf tubes adhered to the microplate lid. Tubes are glued to the lid so that they are coaxially aligned to the culture chambers. The lid is pressed cyclically by an actuator and the compression is transferred to the gels through the reaction tubes. Springs are included so that the lid can return to its original position after the compression (Fig. 3**d**) (Ji *et al.*, 2014). While this system is a simple, compact and inexpensive way to convey compression to 3D cultures, each cell does not experience the same stimulation, which depends on their position inside the chamber. In fact, since the gel is not confined, cells undergo compression or stretch when they are in the lower or the upper part of the well, respectively.

Strain bioreactors

Tensional forces have also been investigated as physical conditioning cues both for 2D and 3D cultures. Typical stretching systems rely on seeded elastomeric membranes that are deformed in an isotropic fashion or following a preferential direction. Possible methods to induce deformation consist of a pneumatic circuit that inflates an air chamber below the culture (Howard *et al.*, 1998) or of a piston-actuator element that pushes on the compliant substrate (Tabatabaei *et al.*, 2014) (Fig. 4**a**). A method to exert uniaxial tension was developed by using a silicone chamber hosting a 2D culture paired with an actuator that cyclically pulls the corners towards opposite directions (Fig. 4**b**) (Hata *et al.*, 2013). Using silicone as a building material allows for easy optical evaluation of the samples without compromising their sterility or interrupting the ongoing culture. This system, even though still limited to 2D cultures, ensures high reproducibility because almost every cell is subjected to similar stimulation (Hata *et al.*, 2013). In case of a 3D culture, the most adopted design choice consists in gripping two sides of the construct with a fixed anchor and a moveable holder so that the motion of the latter induces the stretching of the engineered tissue. These components of the bioreactors are immersed in a chamber containing medium and need an external actuation that induces their motion. Such design has been used to culture hDPSCs on silk fibroin scaffolds for alveolar bone regeneration purposes (Han *et al.*, 2010).

Combined stimulation bioreactors

Depending on the tissue to model or regenerate *in vitro*, multiple physical cues can be applied simultaneously by means of more sophisticated bioreactors. One example was employed by Mathes *et al.* (2010) to mimic the mechanical environment of soft oral tissues. This system combines compression and shear forces and comprises six stainless steel chambers continuously streamed with medium. Collagen sponge scaffolds are held on the bottom of each chamber by swaying holders and a compliant silicone membrane exerts compression when it is pneumatically deformed (Fig. 4**c**). It should be noted that several experimental factors contribute to the outcome: chemical factors, environmental conditions, cell populations, scaffolds chemical and mechanical properties, viscoelasticity and microstructure of the biomaterial, surface topography. Hence, comparing specific results of different studies remains challenging. Moreover, cellular behaviour strictly depends on the parameters that define mechanical stimulation such as frequency, magnitude and duration. All these aspects shall be kept in

consideration when interpreting results obtained from bioreactors.

Microfluidic "organ-on-a-chip" devices

Considerable advances in microfluidics and microfabrication techniques during the last decades resulted in the development of devices able to handle and analyse fluids at the microscale, also termed "lab-on-a-chip" systems. These devices rely on miniaturised circuits that can be provided with reservoirs, pumps and valves. Their main advantages are the reduction of samples and reagents volumes, the high throughput, controlled transport phenomena and portability. Lab-on-a-chip systems can perform basic and complex laboratory operations and assays starting from biological samples and have started to be used for diagnostic purposes. For example, microfluidic devices have been used to analyse saliva and detect both oral and systemic diseases (Salehipour Masooleh *et al.*, 2020).

Organs-on-chips are microfluidic devices used for culturing living cells in continuously perfused, micro-engineered chambers that are increasingly exploited to model the physiological functions of organs and tissues (Bhatia and Ingber, 2014). The goal of these systems is to reproduce basic features of tissue or organ function, going beyond classic 2D monoculture systems. In the last years, the increasing complexity of microfluidic co-culture systems is allowing to more faithfully model real, *in vivo*-like situations (Maoz *et al.*, 2018; Nikolaev *et al.*, 2020). In these set-ups, two or more micro-channels are connected by porous membranes, lined by different cell types on the opposite sides, to recreate tissue-tissue interfaces and to emulate as faithfully as possible the minimal functional units that would recapitulate tissue- and organ-level functions (Bhatia and Ingber, 2014; Huh *et al.*, 2010). These devices are produced by soft lithography (Duffy *et al.*, 1998) and composed predominantly of poly-dimethylsiloxane, a transparent polymer that is bonded on a thin glass plate so that real-time, high-resolution imaging of cultures cells and tissues is feasible (Bhatia and Ingber, 2014). These systems can be engineered to incorporate the several environmental cues that affect tissue and organ functions (Bhatia and Ingber, 2014; Griep *et al.*, 2013; Smith and Gerecht, 2014) and permit analysis of organ-specific responses. These devices have already been used to model, among others, the liver (Sivaraman *et al.*, 2005), kidney (Baudoin *et al.*, 2007), intestine (Kim and Ingber, 2013), lung (Huh *et al.*, 2012), heart (Grosberg *et al.*, 2011), muscles (Grosberg *et al.*, 2012), bone (Park *et al.*, 2012), bone marrow (Torisawa *et al.*, 2014), skin (Atac *et al.*, 2013), blood vessels (Shin *et al.*, 2004), nerves (Park *et al.*, 2013; Shi *et al.*, 2013) and brain-blood barrier (Adriani *et al.*, 2017). Organs-on-chips system are being increasingly employed to model tissue responses to mechanical stimuli and shear stress (Bhatia and Ingber, 2014), electrical activity (Douville *et al.*, 2010), immune responses (Benam *et al.*, 2016; Huh *et al.*, 2010; Kim *et al.*, 2016b), neuronal activity (Achyuta *et al.*, 2013) and whole microbiomas (Kim *et al.*, 2016a; Kim *et al.*, 2016b) as well as for drug testing (Bhatia and Ingber, 2014), including pharmacokinetic and pharmacodynamics studies (Abaci and Shuler, 2015). Finally, the vascular channels of different organ-specific chips can be linked to study multi-organ physiological coupling (Maschmeyer *et al.*, 2015). Chips can be engineered to faithfully reconstitute the microenvironment of the cell/tissue of interest. Fluid flow, cell and extracellular matrix patterning as well as mechanical forces can all be finely regulated and defined to reconstitute a physiological microenvironment that would mimic *in vivo* tissue organisation or, for example, stem cell niches. Similarly, these variables can be altered to assess the response of tissues and cells to specific stimuli in a complex, integrated environment.

Microfluidic devices for the emulation of human dental tissues

Only recently, these systems started to be applied to dental research. Simple microfluidic devices have been used to perform indirect co-cultures and test the influence of different concentrations of factors secreted by human gingival fibroblasts (hGF) and human periodontal ligament stem cells (hPDLSCs) on dental pulp cell populations (Kang *et al.*, 2016). Briefly, the device has two reservoirs containing respectively conditioned medium and basic medium, which are connected to a gradient generator circuit that exploits laminar flow and diffusion to create different factors concentrations and convey each of them to separated culture chambers (Fig. 5**a**). The main advantage is the remarkable high-throughput and the possibility of testing different experimental conditions simultaneously (Kang *et al.*, 2016).

For direct co-culture, chips with different features needed to be introduced. These chips often consist of reservoirs for medium and different parallel channels containing 2D cultures or cell-laden hydrogels. Depending on the lateral connection between channels, it is possible to control the interaction between cell populations. This type of chip was used to emulate tooth innervation *in vitro*. *In vivo*, mouse teeth actively repeal innervation during embryonic development, while they are rapidly innervated few days after birth, at the onset of the mineralisation of dental hard tissues (Pagella *et al.*, 2014a). Co-cultures of trigeminal ganglia with embryonic and postnatal teeth in microfluidic chips faithfully reproduced the *in vivo* situation, for example innervation was constantly repealed over several days of culture by embryonic teeth, while it was promoted and attracted by postnatal teeth (Pagella *et al.*, 2014b). Then, this chip was employed to study the neurotrophic properties of ameloblastomas (Pagella *et al.*, 2020a) as well as to compare the neurotrophic properties of hDPSCs and human bone marrow stem cells, showing that

hDPSCs induce the growth of significantly longer axons as well as the formation of extensive neuronal networks (Pagella *et al.*, 2020b). In this case, narrow grooves between the channels allow only axons to pass and establish a connection between the compartments (Fig. 5**b**).

Biological material other than cells can be introduced in the microfluidic devices. França *et al.* (2020) attempted to create a model of tooth that could mimic the interface between dentine and restorative resins and monitor their interaction with stem-cells-derived odontoblasts in real time. The device has a simple design: it comprises two parallel channels with two chambers that are separated by a decellularised dentine fragment retrieved from a donor. Dentine is inserted in a central groove structure and has the aim to separate the pulp side of the chip, where cells are seeded, and the cavity side, where the biomaterial is inserted (Fig. 5**c**).

Organ-on-a-chip as a novel frontier for drug discovery

Organ-on-a-chip devices are also increasingly used for drug discovery. Drug discovery can be defined as a very complex process needed to identify novel molecules that have the potential to become therapeutic agents. The main goal of the drug discovery process is to recognise a novel molecule that may be useful to address unmet medical needs across different therapeutic fields. Discovery progression includes target identification and validation, hit identification, lead production and optimisation as well as detection of a promising molecule candidate for additional development. The development part includes optimisation of chemical synthesis and its formulation, toxicological studies in animals, clinical trials and ultimately regulatory approval (Mohs and Greig, 2017). Discovery and development processes are time-consuming and very expensive. Most of the time, the drug clinical trial failing may be linked to the lack of predictivity of current preclinical models. Recent studies have established that microfluidics tool have the ability to realise an interactive and dynamic cell microenvironment, extremely important to reproduce cell and organ architectures *in vivo*. For this reason, the microfluidic technology could be extremely useful to identify promising candidate molecule and to evaluate drug concentrations in a more representative and less consuming time (Polini *et al.*, 2014). The combination of 3D cultures with microfluidic technology provides a more suitable culture environment and increase tissue or organ properties, offering a promising novel platform for *in vivo*-like tissue-based methods, such as organ-on-chip systems (Li *et al.*, 2012b).

Microfluidic platforms for pharmacokinetic and pharmacodynamic modelling

These advanced micro-engineered platforms not only simulate the physiological properties at the cellular or organ level, but also could be used to predict human pharmacokinetic and pharmacodynamic responses to drugs, avoiding the use of animal models (Prantil-Baun *et al.*, 2018). During drug development, experiments on cells and animals are performed to assess pharmacokinetic and pharmacodynamic parameters, frequently with the support of mathematical pharmacokinetic and pharmacodynamic models. Microfluidic tools, due to their structure, which integrates several components, can be particularly important for reproducing drug pharmacokinetic, which play a key role in defining the *in vivo* pharmaceutical activity. During *in vitro* drug testing, microfluidic devices could allow for the evaluation of physiological dynamic drug concentration profiles and, thus, help to reproduce *in vivo* drug outcomes more faithfully, decreasing the use of animal models during drug progression. For example, proof-of-concept studies have assessed the capability of microfluidic pharmacokinetic-pharmacodynamic devices to evaluate the dose- and time-dependent effects of doxorubicin and gemcitabine alone or in combination therapy. Such studies have demonstrated the ability of these microfluidic pharmacokinetic-pharmacodynamic devices to model dose- and time-dependent effects of these drugs on cell viability, as well as species-specific (human, mouse) pharmacokinetic profiles (Guerrero *et al.*, 2020). Organ-on-a-chip devices have also been used to model multi-organ pharmacokinetic-pharmacodynamic relationships. In a recent study, a heart-on-a-chip model was fluidically linked to a liver-on-a-chip model to evaluate the temporal pharmacokinetic/pharmacodynamic relationship for terfenadine. Using the data obtained from the device, a mathematical model was developed to predict the effect of terfenadine in preclinical studies (McAleer *et al.*, 2019). Similarly, a microscale cell culture analogue, composed of three cell culture chambers connected with fluidic channels to mimic multi-organ interactions, was used to test the toxicity of an anticancer drug, 5-fluorouracil. Similar to *in vivo* situations, each cell type exhibited differential responses and the responses in the microfluidic environment were different from those in static environments (Sung *et al.*, 2010).

Organ-on-a-chip microfluidic devices and bioreactors are tightly linked to mathematical and computational modelling approaches fundamental for their design, optimisation and readout. Computational analysis is gaining increasing importance to interpret cell behaviour in *in vitro* system at single cell resolution and to understand patterns emerging from highly complex systems such as organ-on-a-chip emulation devices (Del Sol and Jung, 2021). In addition to data interpretation, computational modelling is increasingly used to simulate complex biological processes. For example, recent studies managed to satisfactorily model 3D chondrocyte cell culture growth in a porous scaffold placed in a bioreactor. The simulation predicted in good agreement with experimental evidence the

local shear stresses, glucose concentration and 3D cell growth inside the porous scaffold (Shakhawath Hossain *et al.*, 2015). Modelling has also been successfully applied to optimise experimental designs. For example, in a recent work, computational simulations were used to optimise the geometry and oxygen gradients inside hydrogels used to emulate vascularised bone (Bersini *et al.*, 2016).

Conclusions

The maintenance and regeneration of human dental tissues constitute a great, unmet clinical need. Several attempts have already been made at using dental pulp and periodontal stem cells for regenerative purposes, mostly conducted on animal models (mice, rats, sheep *etc.*) or on simple cell culture systems. Despite some encouraging progresses, dental regenerative therapies are very far from any real clinical application. This is tightly connected with the absence of proper platforms that model human dental pulp in all its complexity and this lack of information and tools is a fundamental reason for the absence of credible pharmacological or cellular therapies for dental diseases. Innovative bioreactor-based and microfluidic organ-on-a-chip culture systems are providing increasingly more faithful tools to emulate human dental tissues. As such, these tools will have a great impact on issues concerning dental public health and design of new, dental-specific drugs. They will also represent a unique tool to assess the side effects that systemic treatments could exert on dental tissues, an often-neglected aspect that nevertheless represents an important burden for global healthcare systems. Thus, these approaches have the potential to advance the dental field, while promoting innovation and reducing dental health-care costs.

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The authors declare no competing interests.

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Discussion with Reviewers

Thomas Diekwisch: Which bioreactor approach will be most suitable for modelling the integrity of dental stem cells and potential epithelial-mesenchymal interactions? Do you think any of the models proposed will benefit dental tissue engineering?

Authors: Epithelial-mesenchymal interactions that lie at the basis of tooth development are very well modelled in classical organotypic cultures, which allow direct contact between the dental epithelium and mesenchyme. Tooth development happens in the absence of mastication-induced high mechanical loads and, therefore, bioreactors that apply significant forces would constitute an unnecessary input for the emulation of epithelial-mesenchymal interactions. Concerning the emulation of "isolated" dental pulp or periodontium, organ-on-a-chip devices represent the most promising design, as they allow for the integration of key elements, such as innervation, vascularisation and immune system, that are necessary to reconstitute a fully functional tissue. Concerning the formation/engineering of enamel, its biological (re)generation seems still far-fetched. This is a highly complex process realised over many years *in vivo* and even minor alterations to any of its aspects can lead to the generation of a defective enamel. Therefore, accelerating this process *in vitro* and obtaining a bona-fide enamel seems at the moment still unplausible.

Reviewer 2: Do the authors think that 3D cultures in spheroids or organoids will help the research in this specific field?

Authors: 3D culture systems are already being applied to model dental tissues (Natsiou *et al.*, 2017) and they will be of great benefit for the field. 3D culture systems provide a much more physiologically relevant geometry to the culture systems and can be implemented within organ-on-a-chip devices. We were recently awarded a research grant for the development of a "tooth-on-a-chip" system, which will include 3D culture elements.

Editor's note: The Scientific Editor responsible for this paper was Juerg Gasser.