Implantation of dental pulp stem cells in a biodegradable scaffold for dental pulp tissue engineering

By

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DEDICATION

To my loving family, my husband Vini, our son Chip and daughters Gabriella and Julia, for their support and unconditional love during my dedication to this project.

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CHAPTER I

1. Background and Significance

For centuries prosthetic appliances were fabricated to replace lost teeth, soft and hard tissue loss (Powers, 2006). A variety of materials have been used, from vulcanite and porcelain teeth to microwavable methyl-methacrylate and highly resistant resin teeth, which were developed and evolved to improve materials properties and biocompatibility (Grant, 1980; Watanabe *et al.*, 1999; Starcke, 1975). Ever improving techniques of prosthesis fabrication have delivered excellent function capability to masticate and esthetics for social acceptance in any environment, restoring for patients what was once lost.

Over the past four decades implant dentistry has been providing exciting possibilities for a "third dentition", enabling great improvement of masticatory function, esthetics, hard and soft tissue maintenance. The development of Guided Bone Regeneration and Guided Tissue Regeneration, CT Scans, CAD-CAM imaging systems and computer technologies greatly enhanced treatments for patients who experienced extreme bone and soft tissue loss. With the advancement of grafting techniques to properly accommodate implant-supported restorations, dental implants gave patients a "third chance" to achieve proper function and esthetics. However, much effort, resources and time are spent on lengthy therapy to construct prosthetic devices that need frequent maintenance and are technique sensitive, with the potential of causing more tissue loss when failure occurs.

Today research is focused in the field of Tissue Engineering as the avenue for the regeneration of lost structures and tissues damaged by trauma, cancer and other diseases.

Advancements in the molecular biology field and stem cell technology are uncovering new horizons in therapeutic possibilities for the tissue and organ replacement.

Tissue engineering uses a combination of three elements: cells, scaffolds and morphogenic signals. Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences to the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ (Langer and Vacanti, 1993).

2. Purpose and Hypothesis

The purpose of this study was to characterize a model for dental pulp tissue engineering that is based on the implantation of dental pulp stem cells in tooth slice/scaffold devices.

The hypothesis of this study is:

Seeding of Human Dental Pulp Stem Cells in a biodegradable scaffold within the pulp chamber of a human tooth slice allows for the engineering of a tissue with cellularity similar to the dental pulp.

Our hypothesis will be tested through the following three specific aims:

Specific Aim 1: To characterize a model system to culture dental pulp stem cells in a biodegradable scaffold inside the canal of a tooth.

Specific Aim 2: To evaluate the cellular proliferation and spatial distribution inside the tooth slice/scaffold system *in vitro*.

Specific Aim 3: To evaluate the morphological characteristics of the dental pulp-like tissue engineered within the tooth slices *in vivo*.

Null hypothesis:

Seeding of Human Dental Pulp Stem Cells in a biodegradable scaffold within the pulp chamber of a human tooth slice does not allow for the engineering of a tissue with cellularity similar to the dental pulp.

3. Literature Review

A. Tissue Engineering

Twenty years ago, Charles Vacanti and Robert Langer from the Harvard Medical School published a classical article describing the concept of tissue engineering. The first recorded use of the term tissue engineering was in an article entitled "Functional Organ Replacement: The New Technology of Tissue Engineering", published in Surgical Technology International in 1991 (Vacanti *et al.*, 1991). Since then, the Tissue Engineering Society (TES) and Journal was founded in 1994 to help guide its purposes and vision for the future of this field. The Tissue Engineering Society was officially incorporated in the state of Massachusetts on January 8, 1996, with its founding Presidents Drs. Charles and Joseph Vacanti, as well as Dr. Robert Langer of MTI (Vacanti, 2006).

A pediatric orthopedic surgeon at the Children's Hospital, W.T. Green, performed a number of experiments in the early 1970's to generate new cartilage using chondrocytes seeded onto bone spicules and implanted in nude mice (Langer *et al.,* 1991). Although unsuccessful with the experiment, he correctly concluded that with innovative biocompatible materials, it would be possible to generate new tissue by seeding viable cells onto scaffolds. Later at Massachusetts General Hospital and M.I.T., Dr. Burke and Dr. Yanas generated a skin substitute using a collagen matrix to support the growth of

dermal fibroblasts. Dr. Howard Green transferred sheets of keratinocytes onto burn patients, while Dr. Eugene Bell seeded collagen gels with fibroblasts (Bell *et al.*, 1991).

In the mid 1980's, Drs. Joseph Vacanti and Robert Langer designed and implemented scaffoldings synthetically fabricated in the laboratory that had physical and chemical properties that could be manipulated; they were biocompatible and biodegradable polymers as carriers of viable cells. Their original article describing the new technology was published in 1988 in Archives of Surgery (Vacanti, 2006) based on a keynote presentation given at the American College of Surgeons Meeting.

The trilogy of tissue engineering is cells, morphogenic signals, and a scaffold (Langer and Vacanti, 1993). Suitable biochemical factors, namely growth factors, give the morphogenic signals to the cells that will improve or replace biological functions within the newly formed tissue.

B. Engineering of Tooth Structures

In 2002, Young and collaborators dissociated porcine third molar tooth buds into single cell suspensions, and seeded onto biodegradable polymers, letting them grow in rat hosts for 20 to 30 weeks. The results were recognizable tooth structures containing dentin, odontoblasts, a well-defined pulp chamber, putative Hertwig's root sheath epithelia, cementoblasts and morphologically correct enamel organ, containing fully formed enamel. Their study demonstrated a successful generation of tooth crowns from dissociated tooth tissues, and they suggested the presence of epithelial and mesenchymal stem cells in porcine third molar tissues (Young *et al.*, 2002).

C. Stem Cells

The "Toti Potenti" embryonic stem cell generated much ethical concerns when used in laboratory experiments. With further research, a generation of stem cells were found in several tissues of adult humans, offering an excellent alternative for the exploration of stem cell therapy with similar differentiation characteristics when compared to embryonic stem cells, without the ethical concerns associated with it.

Adult stem cells have unique characteristics. They exist as undifferentiated cells and maintain this phenotype by the environment and/or the adjacent cell populations until they are exposed to and respond to the appropriate signals, have the ability to self-replicate for prolonged periods and maintain their multiple differentiation potential throughout the life of the organism. Progenitor cells retain the differentiation potential and high proliferation capability, but have lost the self-replication property unlike stem cells (Barry 2003).

Bone marrow stem cells are being used for marrow transplants in cases of leukemia and some other cancers (Tabbara *et al.*, 2002). Current investigation is being studied for direct injection of stem cells into the heart after myocardial infarction to produce cardiomyocytes (Stamm *et al.*, 2003). Stem cells have also been applied in the regeneration of liver (Petersen *et al.*, 1999), bone (Derubeis and Cancedda, 2004), kidney (Imai and Ito, 2002), and neurons in the central nervous system (Okano, 2002).

Muscle-derived stem cells are being used to regenerate muscle, bone and blood cells.

Mesenchymal stem cells are capable of specific migration to sites of injury, for example, in bone fractures (Mosca *et al.*, 2000; Devine *et al.*, 2002), myocardial infarction (Shake *et al.*, 2002), ischemic cerebral injury and knee joint injury (Murphy *et al.*, 2003). They are also used in skin grafts and vascular grafts (Gronthos *et al.*, 2002). The mechanisms that guide homing of injected or implanted cells, however, remain unclear.

There is much interest on the utility of cell and gene therapy for regeneration (Pomerantz and Blau, 2004; Wagers and Weissman, 2004).

D. Dental Pulp Stem Cells

Stem cell niches were identified in a number of tissues including skin, hair follicles, bone marrow, intestine, brain, pancreas and more recently, in dental pulp (Liu *et al.*, 2006). This offers an excellent alternative to the exploration of multi potent cells capable of being isolated, cultured *in vitro*, and differentiated into other cells and tissues such as dentin, bone, smooth muscle, neural, endothelial and adipose tissue, without the ethical concerns associated with embryonic stem cell research. Dental pulp stem cells were collected from clinically extracted human teeth and enzymatically released from the pulp tissue (Liu *et al.*, 2006).

Paul Sharpe's group at the GTK Dental Institute in London has been studying the interactions of non dental cell derived mesenchyme and embryonic oral epithelium that stimulated odontogenic response in the stem cells (Ohazama *et al.*, 2004) (Sharpe and Young, 2005). A group from the Craniofacial and Skeletal Diseases Branch of the National Institute of Health-USA was able to identify in adult human dental pulp, in human primary teeth and in periodontal ligament, stem cells that were highly clonogenic and able to be

cultured *in vitro,* forming cell clusters (Gronthos *et al.*, 2000). These cells were expanded *ex vivo* and expressed a heterogeneous assortment of markers associated with mesenchymal stem cells, dentin, bone, smooth muscle, adipose and neural tissue. They are often found in highly vascularized sites. A study, using several markers of the microvasculature networks, has suggested that pulp stem cells are intimately associated with the blood vessels of pulp tissue, especially pericytes and smooth muscle cells (Shi and Gronthos, 2003). Both supportive connective tissues of bone marrow and dental pulp contain stromal stem cell populations with high proliferative potential, capable of regenerating their respective microenvironments with remarkable fidelity, including the surrounding mineralized bone and dentin (Gronthos *et al.*, 2000; Gronthos *et al.*, 2002). The demonstration of specific markers of dental pulp stem cell differentiation pathways is still under investigation. Also, their characteristics and gene expression patterns *in vitro* might be different than *in situ.*

Dental pulp stem cells were able to differentiate and secrete a matrix that produces osteogenesis and dentinogenesis *in vitro* and *in vivo*, with small teeth produced *in vivo*, but with a disorganized dentinal-pulpal complex (Miura *et al.*, 2003). This could be related to the inability of these cells to produce adequate and organized vascularized network *in vitro* (Tepper *et al.*, 2003).

E. Growth factors relevant for pulp biology

In the angiogenic process of the pulp of mature teeth, new capillary formation was identified as occurring directly underneath the odontoblastic layer, through the regulatory effect of growth factors. Examples of growth factors involved in pulp biology are several members of the transforming growth factors (TGF-ß), the bone morphogenic proteins

(*Bmp*-2, 4, 7, 11); fibroblast growth factor (FGF); insulin growth factor (IGF); vascular endothelial growth factor (VEGF), and others, expressed by odontoblasts, fibroblasts and other cell types, or released from the dentin (Sloan, Rutherford et al. 2000) (Delespesse *et al.*, 1996).

Bone morphogenic proteins (BMP) family members are sequentially involved in embryonic tooth development (Nakashima and Reddi, 2003; Nie *et al.*, 2006). The interactions between epithelium and mesenchyme are essential in tooth development (Nakashima and Akamine, 2005) (Sharpe and Young, 2005). *Bmp 4* from the epithelium induces the mesenchyme to be odontogenic (Ohazama *et al.*, 2005). *Bmp2*, *Bmp4*, and *Bmp7* signals expressed in the enamel knot influence both epithelial and mesenchymal cells and are responsible for the maintenance of the enamel knot and the subsequent morphogenesis of epithelium (Thesleff, 2003) (Reddi, 1998). These signals also regulate the patterning of the tooth crown by influencing the initiation of the secondary knots together with mesenchymal signals such as *Bmp 4* (Mikkola *et al.*, 2002). *Bmp 2*, *Bmp 4*, *Bmp 6*, *Bmp 7*, and *Bmp 11* are also expressed during odontoblast differentiation and *Bmp 4* and *Bmp 5* during ameloblast differentiation (Aberg *et al.*, 1997; Nakashima *et al.*, 1999). Such knowledge has been applied in tissue engineering of tooth structures by several authors (Ohazama *et al.*, 2005) (Nakashima and Reddi, 2003) (Nie *et al.*, 2006) (Zhang *et al.*, 2005).

Dentin matrix contains a cocktail of biologically active molecules with a wide range of effects (Smith *et al.*, 1998). Several growth factors are expressed in the dentin (Finkelman *et al.*, 1990; Cassidy *et al.*, 1997), as well as angiogenic cytokines (Roberts-Clark and Smith, 2000). Release of these growth factors could account for the increased

local angiogenesis observed at sites of dental tissue repair (Baume, 1980); (Smith *et al.*, 1990) (Schroder, 1985) (Roberts-Clark and Smith, 2000), and in the pulps of orthodontically moved teeth, where they might mediate local angiogenic responses (Derringer *et al.*, 1996; Derringer and Linden, 1998).

F. Scaffolds

Scaffolds provide a physicochemical and biological three-dimensional microenvironment for cell growth and differentiation, promoting cell adhesion, and migration (Nakashima and Akamine, 2005). The scaffold serves as a carrier for morphogens in protein therapy and for cells in cell therapy. Scaffold material should allow for transport of nutrients, oxygen, and waste. It should be gradually degraded and replaced by regenerative tissue, retaining the feature of the final tissue structure. They should be biocompatible, nontoxic and have proper physical and mechanical strength. Natural polymers such as collagen and glycosaminoglycan offer good biocompatibility and bioactivity, and synthetic polymers can emulate physicochemical features such as degradation rate, microstructure, and mechanical strength. Commonly used synthetic materials are poly-L-lactic acid (PLLA), poly-glycolic acid (PGA), and their copolymers, poly-lactic-co-glycolic acid (PLGA). Scaffolds containing inorganic compounds such as hydroxyapatite and calcium phosphate are used to enhance bone conductivity (Jadlowiec *et al.*, 2003).

A major parameter in tissue regeneration is the choice of a suitable carrier. Ceramics, polymers and collagen matrices have all been used as substrates to culture dental pulp cells *in vitro* and *in vivo* (Zhang *et al.*, 2006). Among these, calcium phosphate materials have been widely used because of good biocompatibility and their ability to support the

formation of hard tissue. Hydroxyapatite, calcium-ß-glicerophosphate and α or ß-tricalcium phosphate were proven able to induce hard tissue formation when used as pulp-capping agents (Tziafas *et al.*, 2002). Titanium fiber mesh is a highly biocompatible material, and even though it is not biodegradable it was not shown to be disadvantageous for hard tissue applications. Titanium fiber mesh, which has been used for bone regeneration, also supported the attachment, growth and differentiation of dental pulp cells (Zhang *et al.*, 2006). Titanium as well as calcium phosphate ceramic exerted little influence on the differentiation pattern of the cells toward an odontoblastic phenotype (Zhang *et al.*, 2005).

Different scaffolds can stimulate different behavior from the same cell population due to its chemical and physical properties, and spatial availability, having an important role in the engineering of tissues (Zhang *et al.*, 2006).

G. *In vitro* and *in vivo* studies on dental pulp regeneration

Cultured adult dental pulp stem cells (DPSC) demonstrated the ability to generate a dentin/pulp-like complex *in viv*o when co-transplanted with hydroxyapatite and tri-calcium-phosphate (HA/TCP) particles subcutaneously into immunocompromised mice (SCID mice) (Shi and Gronthos, 2003) (Shi and Gronthos, 2003).. Typical DPSC transplants developed areas of vascularized pulp tissue surrounded by a well-defined layer of odontoblast-like cells, aligned around mineralized dentin with their processes extending into tubular structures. In addition, orientation of the collagen fibers within the dentin was characteristic of ordered primary dentin, perpendicular to the odontoblast layer (Shi and Gronthos, 2003).

Adult vasculogenesis was demonstrated in mice to be initiated as a response to local tissue ischemia that releases growth factors such as VEGF and others, responsible to mobilize endothelial progenitor cells (Tepper *et al.*, 2005). *In vivo* stimuli to the hypoxic implant site and the wound healing process will stimulate microvascular formation inside and around the scaffold/tooth structure, maintaining and remodeling the newly formed tissue (Tepper *et al.*, 2005).

Recent data suggest that the capacity and potential for adult stem cells to differentiate into a wider spectrum of phenotypes, *'stem cell plasticity'*, is caused by fusion of stem cells with endogenous tissue-specific cells (Camargo *et al.*, 2004; Wagers and Weissman, 2004). Huang *et al.* characterized the human adult dental pulp cell differentiation potential grown in mechanically and chemically treated dentin and stimulated with dexamethasone that produced mineral nodules. They appeared to establish an odontoblast-like morphology, with cytoplasmic processes extending into dentinal tubules revealed by scanning electron microscopy analysis. Their data suggest that isolated human pulp stem cells may differentiate into odontoblasts on dentin *in vitro* (Huang *et al.*, 2006).

Tran-Hung *et al.* reported that Human Pulp Fibroblasts from third molars co-cultured with human umbilical vein endothelial cells induced the organization of endothelial cells into forming tubular structures corresponding to capillaries *in* vivo (Tran-Hung *et al.*, 2006). The direct contact between both cell types was not necessary to induce angiogenesis. The observed effect was due to soluble factors, confirmed with neutralizing antibodies against FGF-2 and VEGF, altering its effect once neutralized.

These results suggest that pulp fibroblasts secrete angiogenic factors, necessary for pulp healing (Tran-Hung *et al.*, 2006).

Another study reported co-expression of desmossomal proteins and vimetin in a specific mesenchymal phenotype (Sawa *et al.*, 2005). This study investigated the expression of vimetin-binding desmossomal proteins in human dental pulp fibroblasts and odontoblasts. Through confocal laser-scanning microscopy, the difuse distribution of desmoplakin (DPK) was demonstrated in the cytoplasm throughout the odontoblastic processes. Dental pulp fibroblasts and odontoblast-like cells that were cultured had mRNA expression of osteocalcin (OCN) and dentinsialophosphoprotein (DSPP) established in the differentiation medium (Sawa *et al.*, 2005). The pulp fibroblasts become odontoblast-like cells expressed vimetin, but not desmoplakin before culturing in the differentiation medium. This suggests that dental pulp fibroblasts that usually express desmoplakin mRNA, and that the desmoplakin 1 production and the bonding of vimetin to desmoplakin 1 occur when dental pulp fibroblasts (DFP) are differentiating into odontoblast-like cells (Mooney *et al.*, 1996; Sawa *et al.*, 2005). Fibroblasts may help induce the formation of an engineered pulp tissue, along with human dental pulp stem cells, placed inside a scaffold in the pulp chamber of a sliced tooth, *in vivo*.

H. The Odontoblast Phenotype

The odontoblast phenotype has been mainly characterized by biochemical analyses of the dentin matrix that correspond to terminal differentiation markers. Odontoblasts secrete type 1 collagen and other non collagenous proteins such as osteonectin, osteopontin, bonesialoprotein, dentin phosphoryn, dentinsialoprotein and dentin matrix protein 1

(Priam *et al.*, 2005). This group analyzed the phenotypes of three independent clonal cell lines from first molar tooth germs of day 18 mouse embryos with RT-PCR and Western Blot. These clones synthesized dentinsialoprotein (DSP), dentin matrix protein 1 (DMP-1) and other extracellular matrix proteins typical of the odontoblasts. However, gene expression patterns and signaling pathways underlying odontoblast differentiation are still unclear (Priam *et al.*, 2005). In a study of differentiation potential of rat dental pulp cells, the authors state that dentinsialoprotein (DSP) and dentinphosphoprotein (DPP) form the major part of dentin noncollagenous proteins (Zhang *et al.*, 2005). The expression of dentinsialoprotein (DSP), an odontoblast-specific marker, was restricted to the outer pulp layer containing mature odontoblasts and was absent in fibrous tissue, nerve bundles and blood vessels (Shi and Gronthos, 2003).

CHAPTER II

1. Abstract

Dentistry has no adequate therapeutic strategies for a number of conditions involving pulp necrosis, especially in young permanent teeth with incomplete root formation. These teeth might benefit from the regeneration of dental pulp tissue thereby allowing for the completion of root formation. The objective of this study was to develop and characterize a new strategy involving tissue engineering based approaches and stem cells to regenerate a viable dental pulp tissue. Freshly extracted human teeth were cut into 1 mm thick cross sections. The pulp tissue was removed and a synthetic biodegradable scaffold made with Poly-L-Lactic-Acid (PLLA) was cast in the root canal space of the tooth slice. These tooth slice/scaffold devices were disinfected in grades of ethanol, treated with 10% EDTA, washed in 1X PBS, and were immediately seeded with human dental pulp stem cells (DPSC) and/or human dental pulp fibroblasts (DPF). Cellularity was evaluated after 1 to 7 days of cell culture *in vitro*. In tooth slices seeded with DPSC and DPF, we observed cellular processes extending into the dentinal tubules, suggestive of odontoblastic differentiation. This model system was also tested *in vivo*. Tooth slices containing human dental pulp stem cells and/or human dental pulp fibroblasts were implanted in the subcutaneous dorsal region of severely compromised immunodefficient mice (SCID mice). Mice were euthanized after 21 days and samples analyzed by histology with H&E staining and immunohistochemistry for Factor VIII to evaluate dental pulp vascularization. Histological examinations showed that the co-implantation of DPSC and DPF in tooth slices allowed for the engineering of a dental-pulp like tissue. We conclude that dental pulp stem cells might be useful to engineer a dental pulp-like tissue in young permanent teeth with incomplete root formation in the future.

2. Introduction

Despite the high success rate of root canal therapy (97% after 8 years follow up), many teeth are not restorable due to apical resorption and fracture, incompletely formed roots or carious destruction of coronal structures (Salehrabi and Rotstein, 2004). For four decades, implant dentistry has provided a "third dentition" to patients who suffered the loss of teeth and soft tissues by surgically and prosthetically using titanium fixtures placed inside bony structures of the patient's mouth and face. Patients with severe bone loss in several areas of the mouth represented serious limitation for this treatment. This motivated the search for solutions to properly place implants in restorable areas, leading to the development of graft materials as well as Guided Bone Regeneration and Soft Tissue Regeneration.

Today we take a step further as we look at the tooth as the first organ that might be feasible to engineer (Sharpe and Young, 2005; Zhang *et al.,* 2005; Nör, 2006). With recent advances in molecular biology sciences and regeneration strategies with stem cells, the application of biologically based tissue engineering approaches to promote regeneration of tooth structures might provide exciting novel therapeutic possibilities in the restorative and endodontic fields (Tziafas, 2004).

3. Materials and Methods

A. Cell Culture

Dental Pulp Stem Cells (gift from Dr. Songtao Shi, University of Southern California) were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA), supplemented with 10% Fetal Bovine Serum (Gibco), 275 units/ml Penicillin and Streptomycin in a humidified CO_2 incubator at 37 ^{0} C. Cell culture medium was changed every other day. When cells were confluent, they were detached from the 75ml flasks with Trypsin (Gibco), centrifuged, and passed at a proportion of 1:3. These cells remain active until approximately passage 20, undergoing senescence after that. All experiments were performed with cells between passage 5 and 8.

B. Preparation of tooth slices:

Freshly extracted human third molars were collected from patients aged 15 through 28, undergoing surgical extractions in the Oral Surgery Clinic at the School of Dentistry – University of Michigan for orthodontic reasons. The teeth were transported in low glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA), supplemented with 10% Fetal Bovine Serum (Gibco), 275 units/ml Penicillin and Streptomycin to the laboratory, cleaned of gingival tissue, PDL and debris with sterile periodontal scalers, and prepared to be cut in 1.0 mm crossectional slices using a diamond blade 303 Series (MK –303 Professional – MK DIAMOND PRODUCTS INC., Torrance, CA) in the Isomet Low Speed Saw (Model 650, South Bay Technology, INC. Japan), cooled with 1X PBS (Phosphate Buffered Saline; Cellgro, Mediatech, Inc.

Herndon, VA). The original pulp tissue was removed from the slice with a sterile #12 Bard Parker scalpel, preserving the pre-dentin area. Each cut slice was placed immediately in 12 well plates containing 1ml per well of PBS-1X - Phosphate- Buffered Saline (Cellgro - Mediatech, Inc. Herndon, VA) and maintained at 4^{0} C. All procedures were performed under aseptic conditions to avoid contamination. Collection of tooth slices was performed under a University of Michigan approved IRB protocol.

C. Scaffold Fabrication Inside the Tooth Slices:

A solution of five percent of Poly-L-lactic acid (PLLA) scaffolds were prepared inside the pulp chamber tooth slices. The detailed description of this protocol is in the Appendix section. Tooth slice/scaffolds were rehydrated in 100% ethanol for 10 min, 90% ethanol for 10mim, 80% ethanol for 10 min, 70 % ethanol for 10 min. This was followed by an incubation in sterile 1X PBS for 1 hour, and a second incubation in 1X PBS overnight at 4^{0} C. The next day, samples were treated with 10% EDTA for one hour, then received three washes with fresh sterile 1X PBS for 30 min each, to be ready for cell seeding procedures.

The tooth slice/scaffold devices were prepared from freshly extracted teeth, cleaned and cut crossectionally as shown in Figure 1. The dental pulp was removed (Figure 1 D) and a PLLA scaffold was cast in the pulp chamber (Figure 1 E, F).

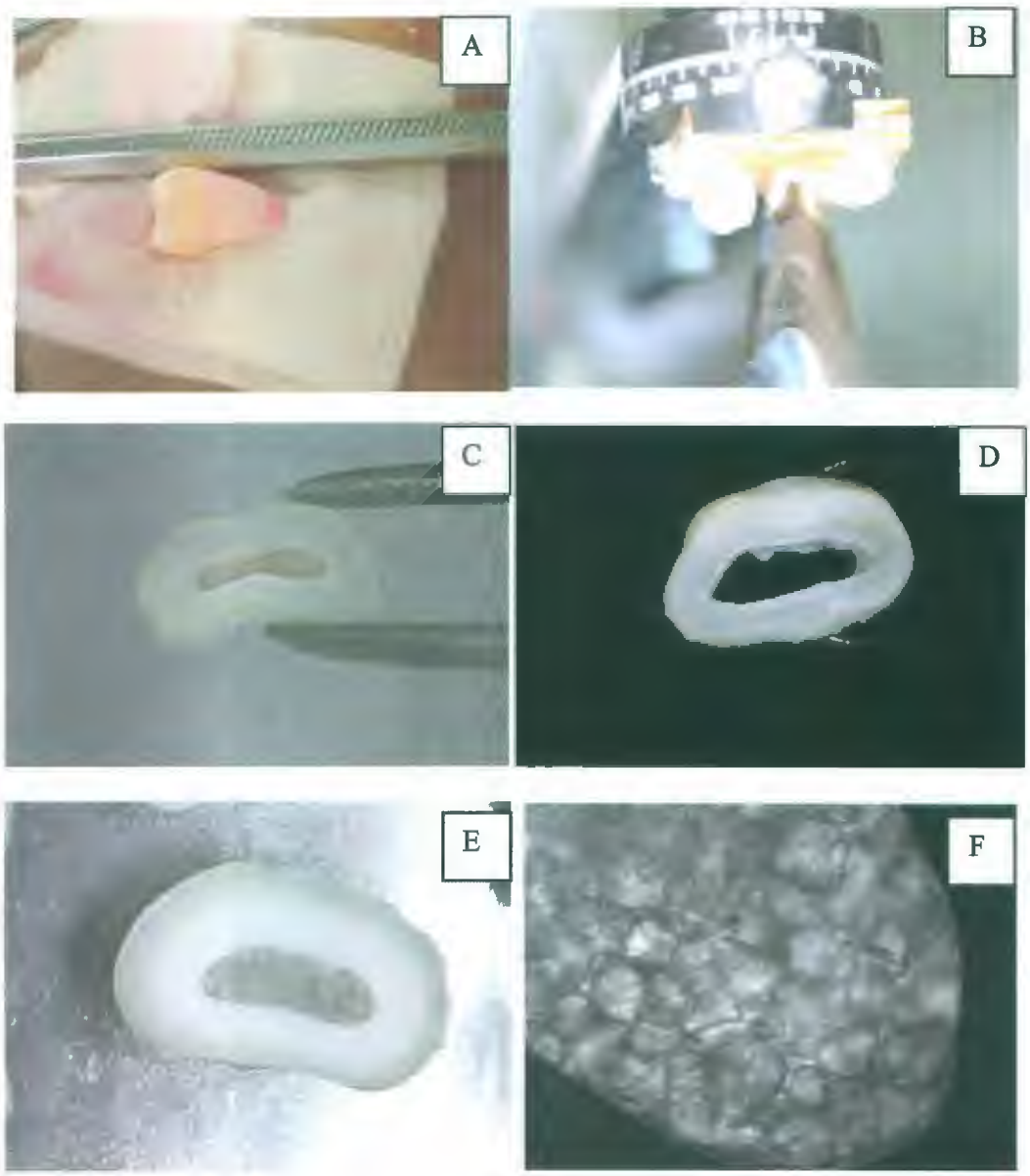

Figure 1: (A) and (B) Freshly extracted teeth are cut into 1mm tooth-slices at the CEJ area. (C) and (D) The original pulp tissue is removed preserving the pre-dentin. (E) PLLA scaffolds are fabricated inside the pulp chamber space. (F) Bright field image of tooth slice/scaffold, 200X.

D. Cell Seeding inside the tooth-scaffold devices

The surface area and volume of scaffold inside the teeth were calculated to determine the number of cells needed per sample. Our standard scaffold is 6x6x1mm. In the area of $36mm^3$ we know we should seed $1x10^6$ cells, according to established protocol (Nör *et al.*, 2001). We classified the sliced teeth into small pulp chambers and large pulp chambers, seeding approximately $5x10^5$ for the large pulp chambers and $3.3x10^5$ for the small pulp chambers (Figure 2).

Cells are trypsinized, detached from the flasks, and centrifuged for five minutes at 800 rpm. They are resuspended for cell counting. Fifty micrometers of Trypan Blue Stain is added to 50µm of resuspended cells and are placed in the hemocytometer for counting, under the microscope. Matrigel® was used in a 1:1 proportion along with cell medium in the cell pellet as described by Nör *et al.*, 2001.

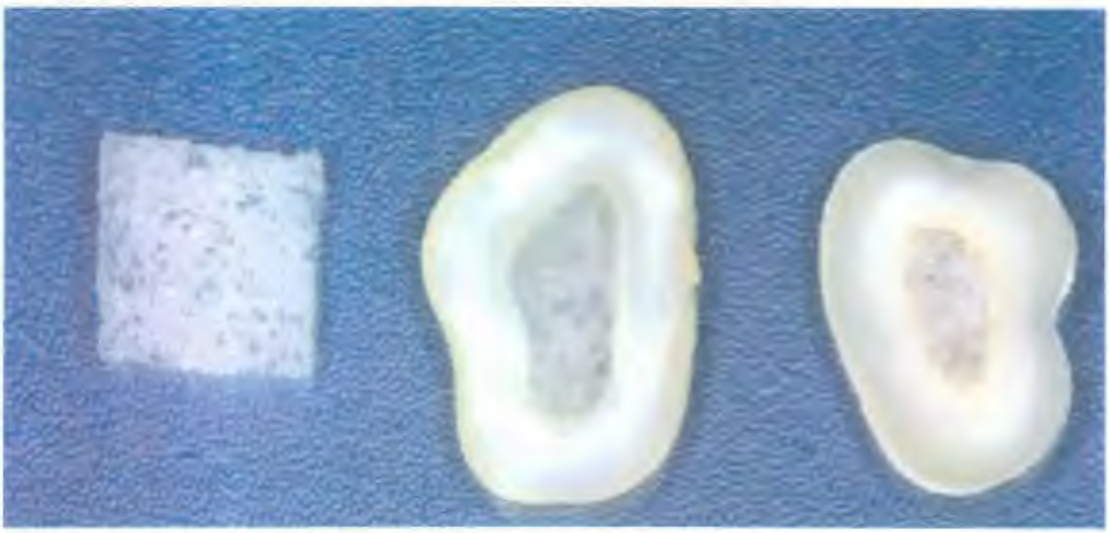

Figure 2: Tooth-slices were classified into large pulp chambers and small pulp chambers. The area and volume are calculated to better estimate the amount of cells we should seed into the different samples. Our standard is the 6x6x1mm scaffold, with $1x10^6$ cells. Large pulp chambers receive $5x10^5$ and small pulp chambers, $3.3x10^5$.

In Vitro **Study**

E. Sample preparation for Image capturing:

A protocol was developed and optimized to determine a concentration of the fluorescent dye that can be used to label cells with minimal toxicity. Long term cell labeling tracers were used to stain dental pulp stem cells (DPSC) in green fluorophore with carboxyfluorescein diacetate succinimidyl ester (CFDA SE®), and simultaneously label dental pulp fibroblasts (DPF) in red fluorophore with carboxy SNARF®1. The tooth-scaffold samples that received pre-stained cells were treated with two 30 min washes of HBSS (Hank's Balanced Salt Solution) without Magnesium and Calcium in the humidified CO_2 incubator at 37 ^{0} C. To evaluate cellularity, a proliferation assay is assessed over time, collecting data at days zero, one, three and five. The samples are assessed for cellular density with samples under inverted microscopy with Leica Sotfware Immaging System, as well as confocal laser microscopy (Nikon). Cell count is performed with manual tag on the pictures using the Image-Pro Software (MediaCybernetics, Inc., Bethesda, MD).

After seven days, images of co-culture in tooth-scaffold devices showing CFDA SE® green cell tracer dye in DPSC and SNARF®1 red cell tracer dye in DPF were obtained with inverted confocal laser microscopy (Olympus), as well as samples that had single cell population with each cell type. Empty controls without cells were also evaluated in this experiment.

In Vivo Study

Severely immunodefficient (SCID) mice (CB.17 SCID; Charles River, Wilmington, MA) were anesthetized with a solution of ketamine®/xylazine®, and received a 1 cm long incision in the dorsum. Bilateral subcutaneous pockets were created to separate the dermis from the underlying muscular layer, as described by Gonçalves *et at.*, 2007. These pockets in the dorsum region received two tooth slice/scaffold devices (one on each side), with the dental pulp cells seeded in the following groups: DPSC alone, DPSC with DPF, DPF alone and empty devices. After 21 days, the mice were euthanized and samples were retrieved for analysis. The use and handling of animals in this study were performed in accordance with UCUCA and ULAM guidelines.

F. Hematoxylin & Eosin Histology

Each sample was fixed in 10% buffered formalin for 24 hours at 4° C, demineralized with 10% formic acid for several days at 4° C, until the dentin didn't offer resistance to being cut for histology. Histological procedures preparing paraffin blocks for histological cuts of 5μm in slides (according to Saw *et al.*, 2004) were prepared for analyses with hematoxylin and eosin staining. Some slides were left unstained for immunohistochemistry (Factor VIII).

G. Immunohistochemistry

Factor VIII Immunohistochemical analysis was performed using DAKO Cytomation EnVision + System-HRP (AEC) with Rabbit Primary Antibodies. Samples were incubated with (F. VIII related antigen Ab-1 Rabbit PAb ref. RB-281-A; antibody diluent- ref. S0809, 1:500) polyclonal rabbit anti-human Von Willebrand Factor for 30 min at room temperature. Sections were washed with 1X PBS, and incubated in the second antibody (Labelled Polymer-HRP, anti-rabbit DAKO, ref. 4008,) for 30min at room temperature. Sections were washed with 1X PBS and AEC substrate chromogen (Dako ref. K 4008) was applied for 5 min. Sections were counterstained with hematoxylin, washed with distilled water and observed under light microscope at 200X magnification.

G. Statistical Analysis

Statistical analysis was performed using One Way ANOVA followed by Tukey Tests for multiple group comparison with SIGMASTAT 2.0 statistical software (SPSS, Chicago, IL, USA). The level of significance was determined at $P \leq 0.05$.

4. Results

In vitro studies

The *in vitro* culture of dental pulp stem cells inside the tooth-scaffold system was observed through the use of fluorescent Cell Tracker Green showing viable cells in a laboratory fabricated environment inside the pulp chamber space of a sliced tooth (Figure 3).

Dentin and PLLA scaffold retained some fluorescent staining and the experiment design was changed to observe cells only in the teeth-scaffold system. We also wanted to be able to tell the cell populations apart (i.e. DPSC and DPF), to understand their spatial distribution within the pulp chamber.

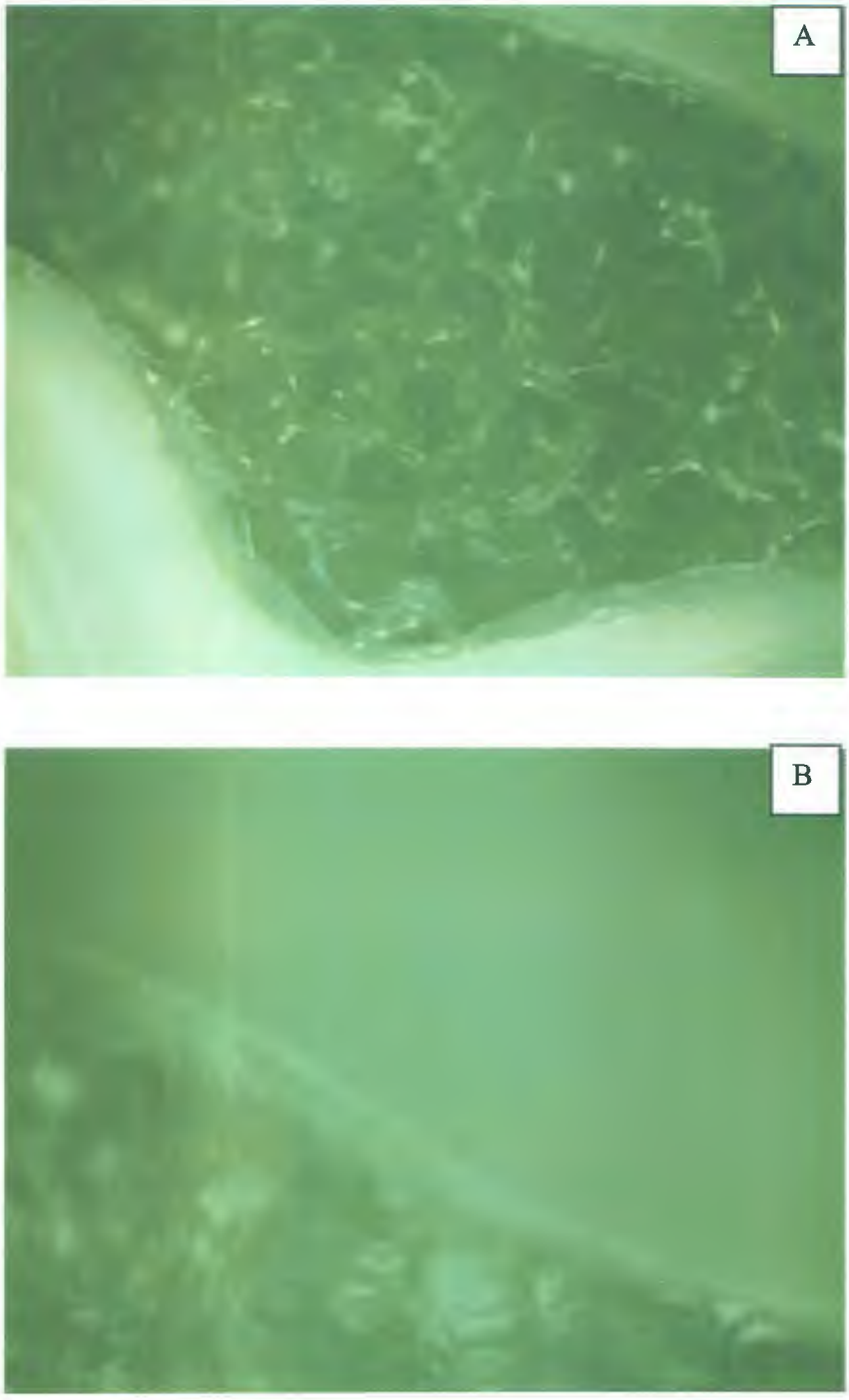

Figure 3: (A) Tooth-scaffold stained with Cell Tracker Green (100X). (B) Empty control shows that dentin and PLLA scaffold retain some fluorescent staining (200X).

For long term cell labeling systems we used carboxyfluorescein succinimidyl ester CFDA SE® and carboxy SNARF®-1 (Molecular Probes, Inc.), which is able to trace cells for up to 7 days *in vitro* (Figure 4).

The cell tracer for dental pulp stem cells (DPSC) was an amine-reactive carboxyfluorescein succinimidyl ester, of color green called CFDA SE®. It passively diffuses into cells, reacts with intracellular amines and forms fluorescent conjugates. The label is inherited by daughter cells after cell division and is not transferred to adjacent cells (Magg and Albert, 2007) (Figure 4A).

SNARF®-1 is the long wavelength fluorescent that we used for the dental pulp fibroblasts (DPF). The cells were pre-stained with the green and red fluorescent probes and then seeded inside the tooth/scaffold device. The experimental conditions tested were: dental pulp stem cells alone, dental pulp stem cells with dental pulp fibroblasts, dental pulp fibroblasts alone and empty control samples. The tooth slice/scaffold devices with single cell population received $5x10^5$ cells, and the dual culture system received $2.5x10^5$ per cell type (Figure 4B).

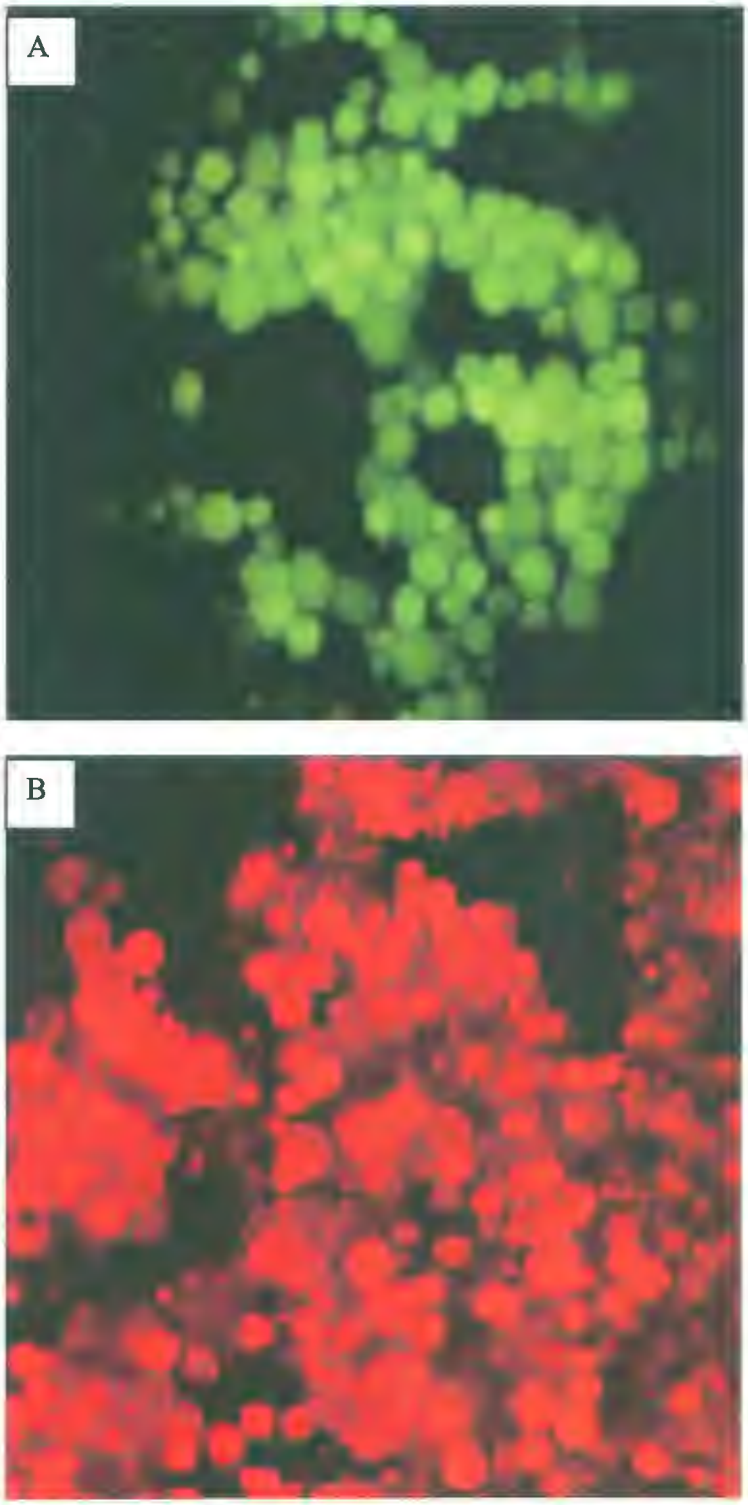

Figure 4: (A) DPSC were stained with CFDA SE® and (B) DPF were stained with SNARF-1®. (400X)

The concentrations of the probes were tested separately for each cell type to determine cell viability and long term maintenance of the immunofluorescent label. DPSC were seeded in 24 well plates (n=12 – $2x10^4$ cells per well) with different concentrations ranging as follows: [25], [20], [15], [10], [5], [2.5] μM

To assess the number of viable cells after they were stained, a Trypan Blue assay was conducted within 24 hours after the dye was applied. Cells were counted with the hemocytometer in the microscope. Data is shown in Figure 5.

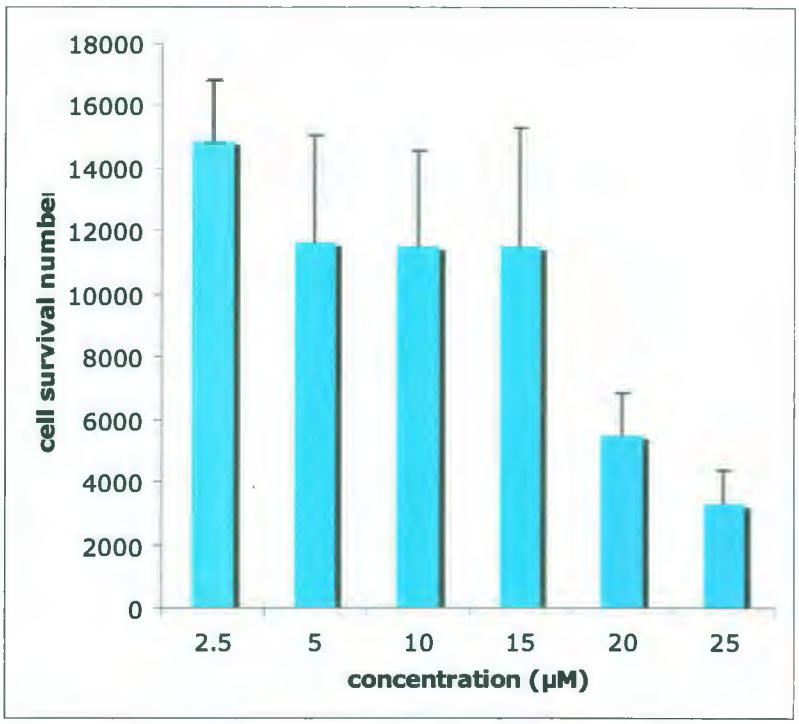

Figure 5: A working concentration of 10μM is found adequate to be used with DPSC for experiments after one week under fluorescent microscopy. Similarly, a working concentration of 1μM was found adequate to be used with DPF.

The proliferation assay was conducted for five consecutive days with 40 samples, and the results are shown in Figure 6. The description of the experiment design for the proliferation assay is to evaluate cell density of different cell groups within the tooth-scaffold devices. The experiment was conducted for five days. Pictures of three random fields of each sample for each cell group was taken at days zero, one, three and five. To obtain pictures, samples were washed twice with 30 min washes of Hank's Balanced Salt Solution (HBSS) for background elimination. After the fluorescence excitation happens in the cells and samples are exposed to UV light, light is emitted through exposure for picture taking and samples can no longer be used. Pictures of three random fields at 400X were taken of each sample, each day.

The cell groups tested in the proliferation assay were: DPSC alone, DPSC with DPF and DPF alone. Each group tested had 12 samples (n=36) plus the empty control group (n=4). Total n=40. The experiment was repeated.

After 5 days, cell density inside the system remained constant when DPSC and DPF were seeded together, *in vitro* (Figure 6). DPSC+ DPF showed a significantly higher cell number after 5 days as compared to the experimental conditions where we had one cell type only (i.e. DPSC or DPF).

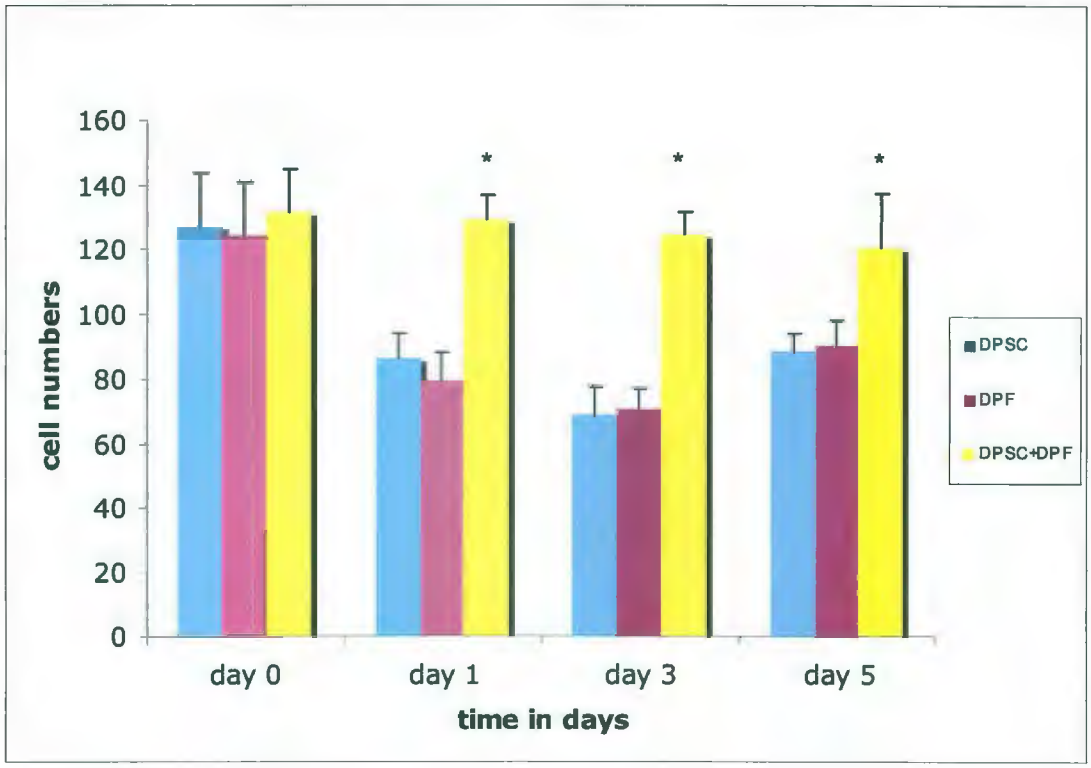

Figure 6: Overall Cell number according to time and culture condition, i.e. DPSC only, DPF only and DPSC + DPF co-cultures. (*, $p < 0.05$).

The confocal laser microscopy was a separate experiment conducted after seven days and had 16 samples. Images were obtained 7 days after seeding suggests morphological changes of DPSC when co-cultured with DPF. DPSC appears to extend cytoplasmic processes inside the surrounding dentinal tubules, suggesting an odontoblast-like morphology (Figures 7 and Figure 8). Such images are not observed in single cell type scaffolds (Figure 9) or empty scaffolds (Figure 10).

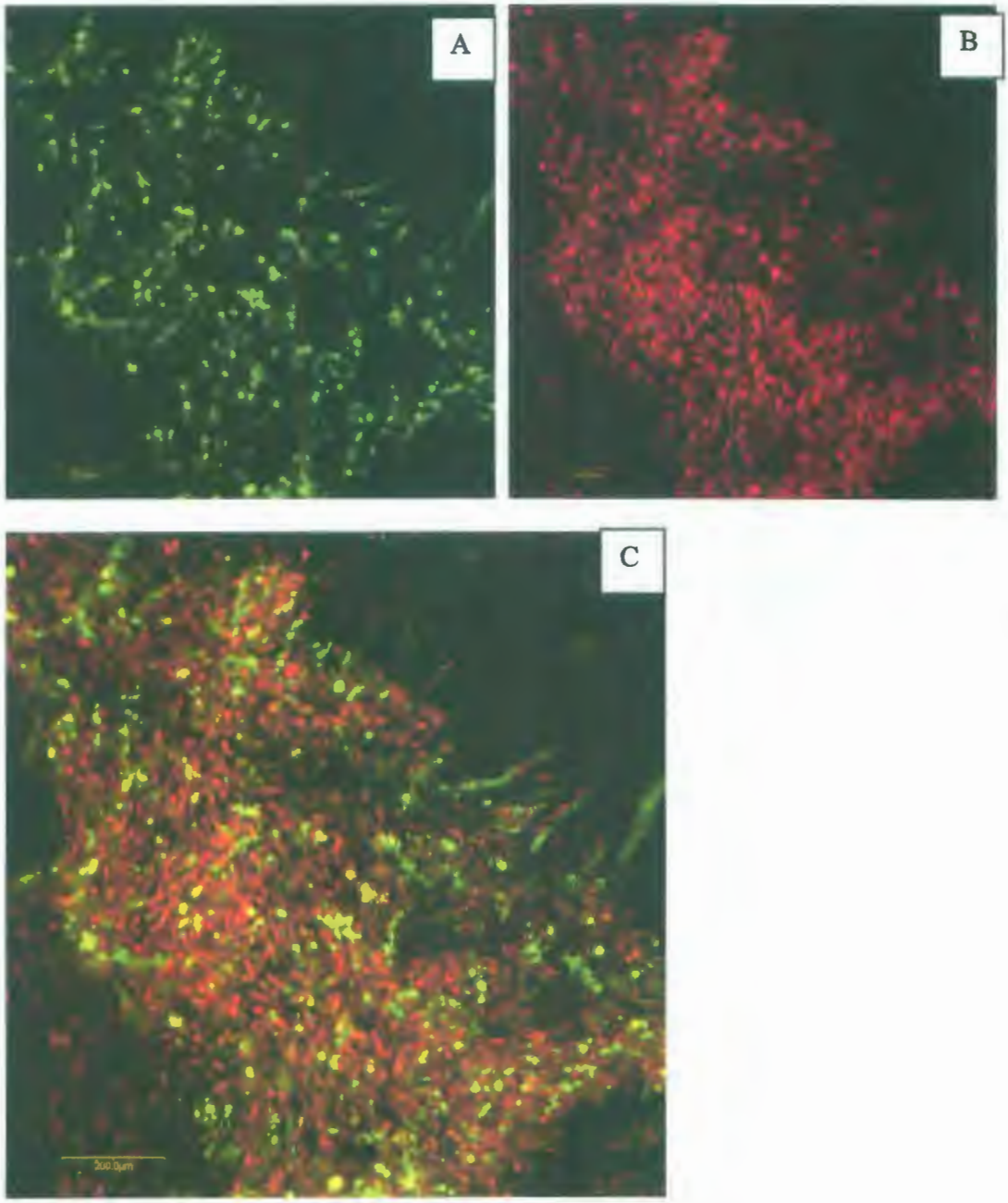

Figure 7: At 7 days *in vitro*, Confocal Laser Microscopy, immunofluorescence of dual stained samples. (A) DPSC stained with CFDA SE®. (B) DPF stained with SNARF® 1. (C) Merged image of both cells on the same sample, showing cellular processes going inside dentinal tubules. 100X. (Total n=16)

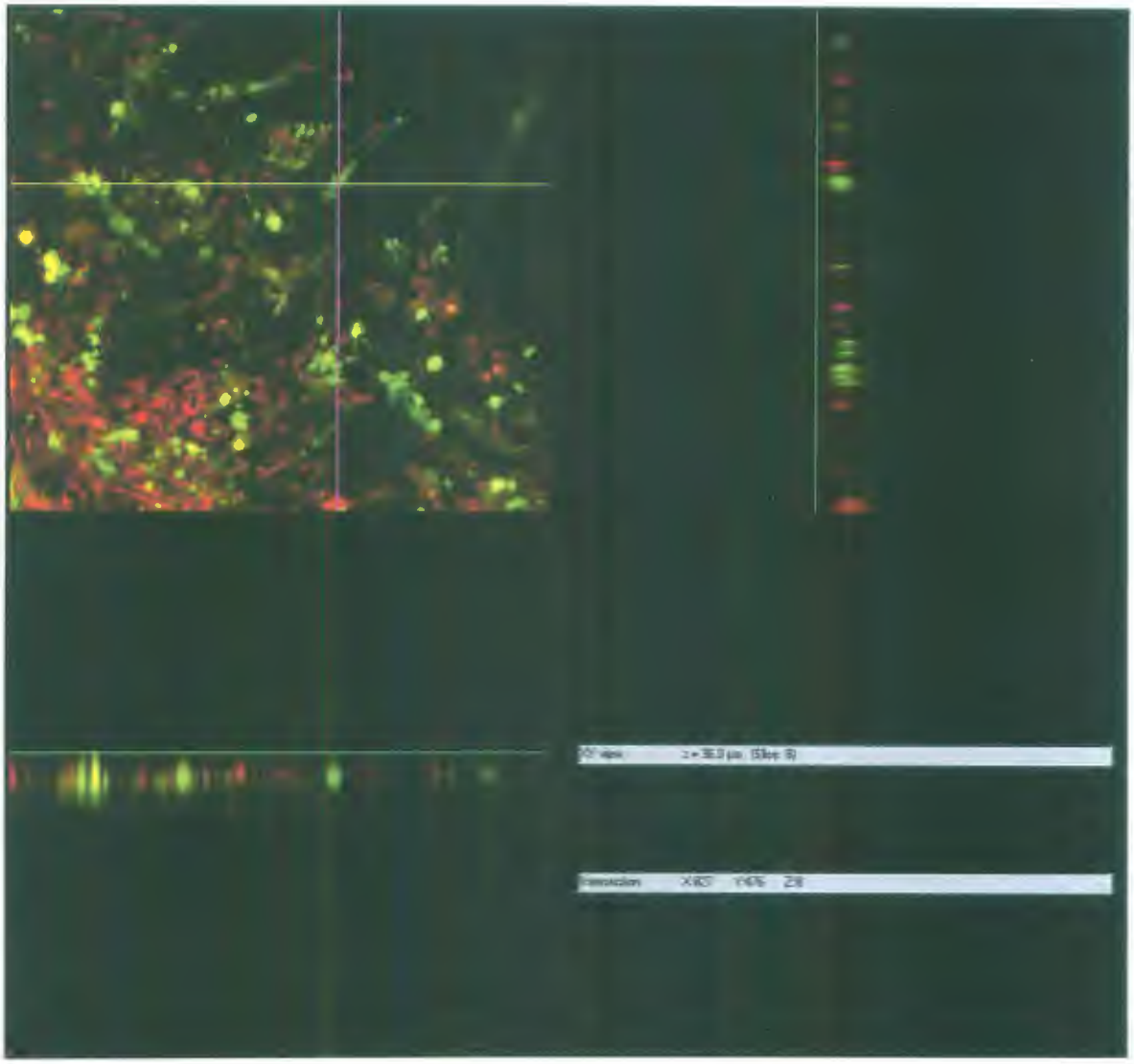

Figure 8: This shows a sliced image of cellular processes in the dentinal area at 7 days *in vitro*, where on the right and bottom images we see with cross section the cells inside the tubules. 400X

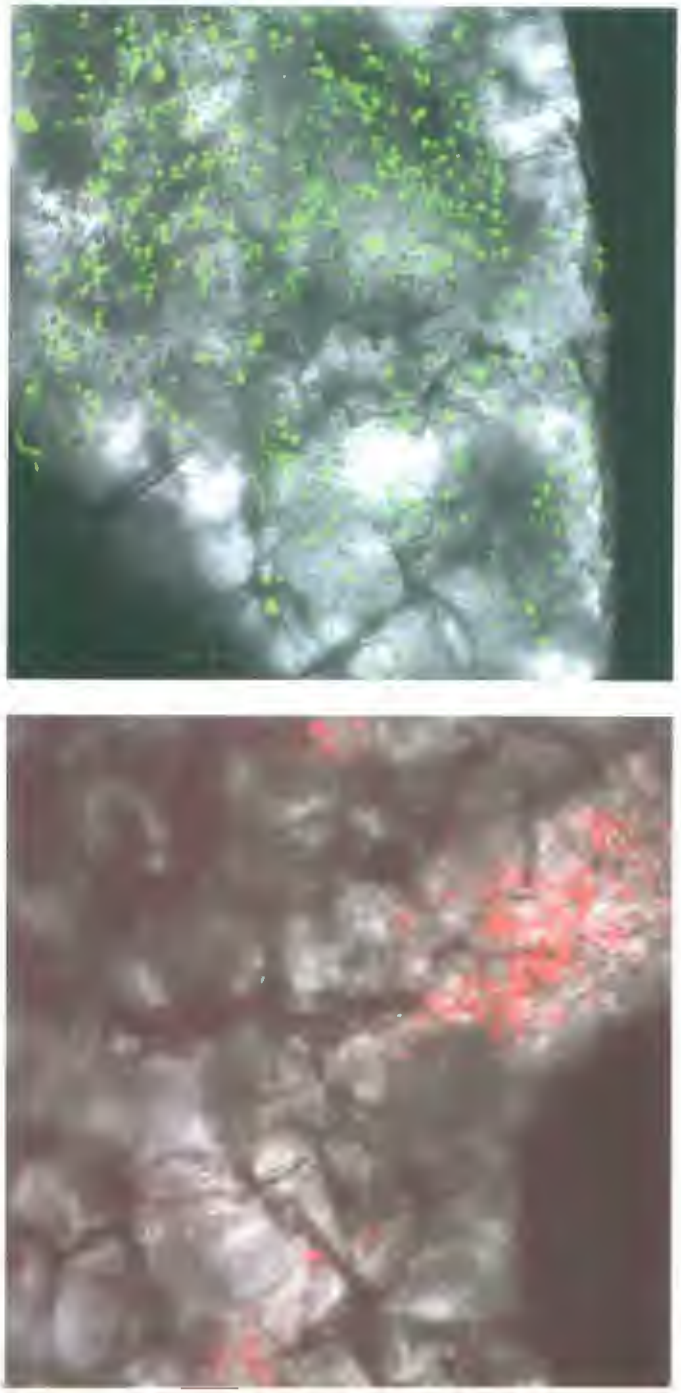

Figure 9: Image of samples with single cell population, where cells were stained with dyes and are shown in bright field background. DPSC in green, DPF in red, 400X.

Figure 10: Empty control sample, with green filter on the top left, red filter on the top right, and bright field on the center. 400X

In vivo studies

Tooth scaffold samples were transplanted in SCID mice (Figure 11 A- E). After three weeks, mice were euthanized and implants were retrieved (Figure 11 F; Figure 12).

Descriptive Histology

Empty tooth-scaffold devices presented histological features of loose connective tissue, well populated with fibroblast cells, with a moderate amount of blood vessels (Figure 13 C). The DPF group presented loose connective tissue formed, fairly vascularized (Figure 13 B). The DPSC group presented with loose connective tissue formation (Figure 13 D). Finally, the DPSC+DPF group had a denser and more fibrous connective tissue present (Figure 13 E).

The present study formed tissue *in vivo* inside the pulp chamber of sliced teeth, seeding dental pulp stem cells, dental pulp fibroblasts, both cells and no cells into a biodegradable PLLA scaffold that was built inside the pulp chamber space of the sliced tooth. The cells that lined the pre-dentin area had some morphological resemblance to the odontoblastic layer as found in normal dental pulp (Figure 13 A). These cells were counted under light microscopy. We observed that the average number of cells lining the pre-dentin is significantly higher in the group with DPSC and DPF, as compared to the DPSC alone, DPF alone and Empty Control experimental conditions evaluated here (Figure 14).

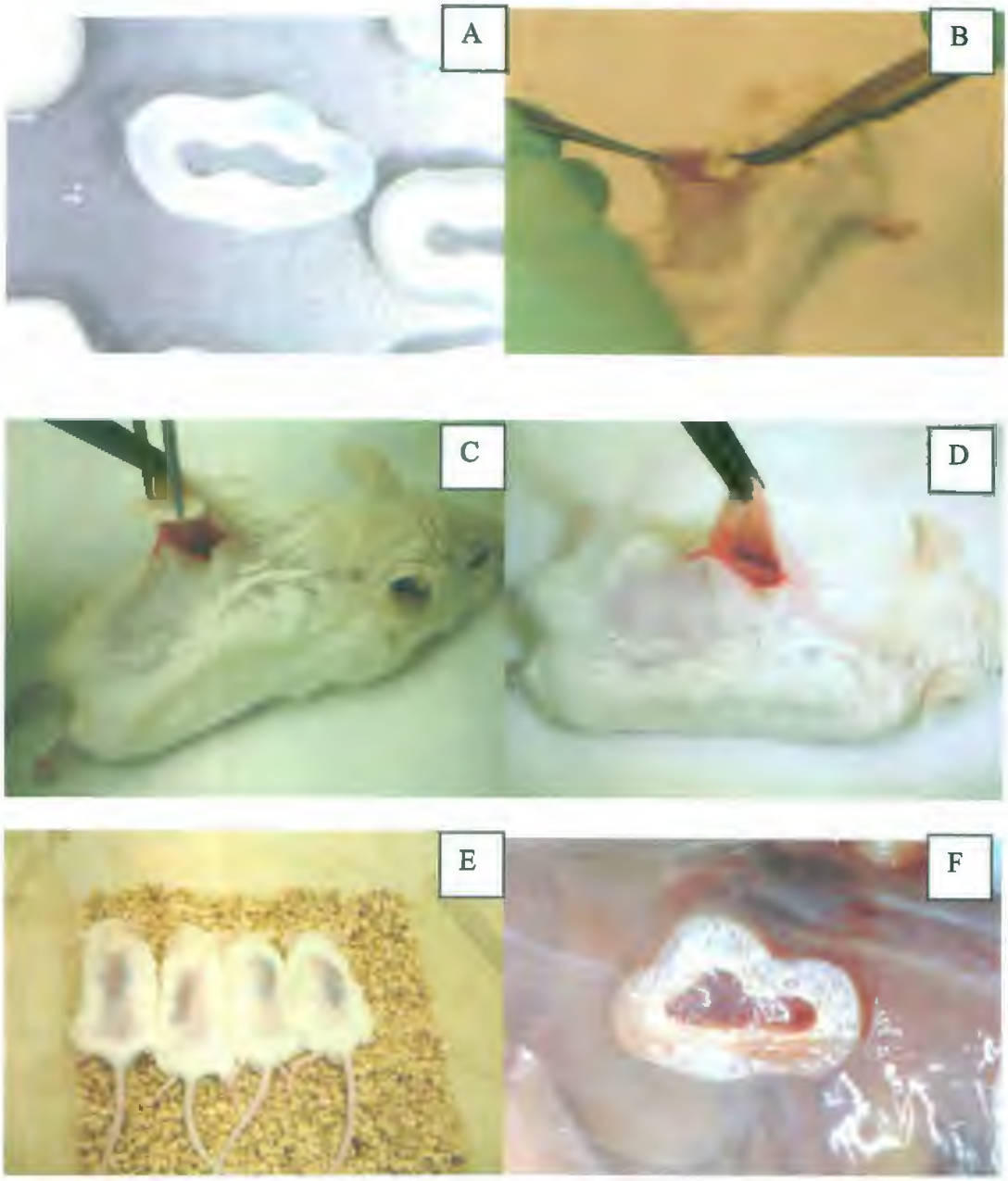

Figure 11: Tooth scaffold devices were prepared and seeded with cells. Four groups were tested: (A) Empty controls, (B) DPSC, (C) DPF, (D) DPSC+DPF (total n=16).
The experiment was repeated (total n=16). (F) Samples were retrieved after three weeks.

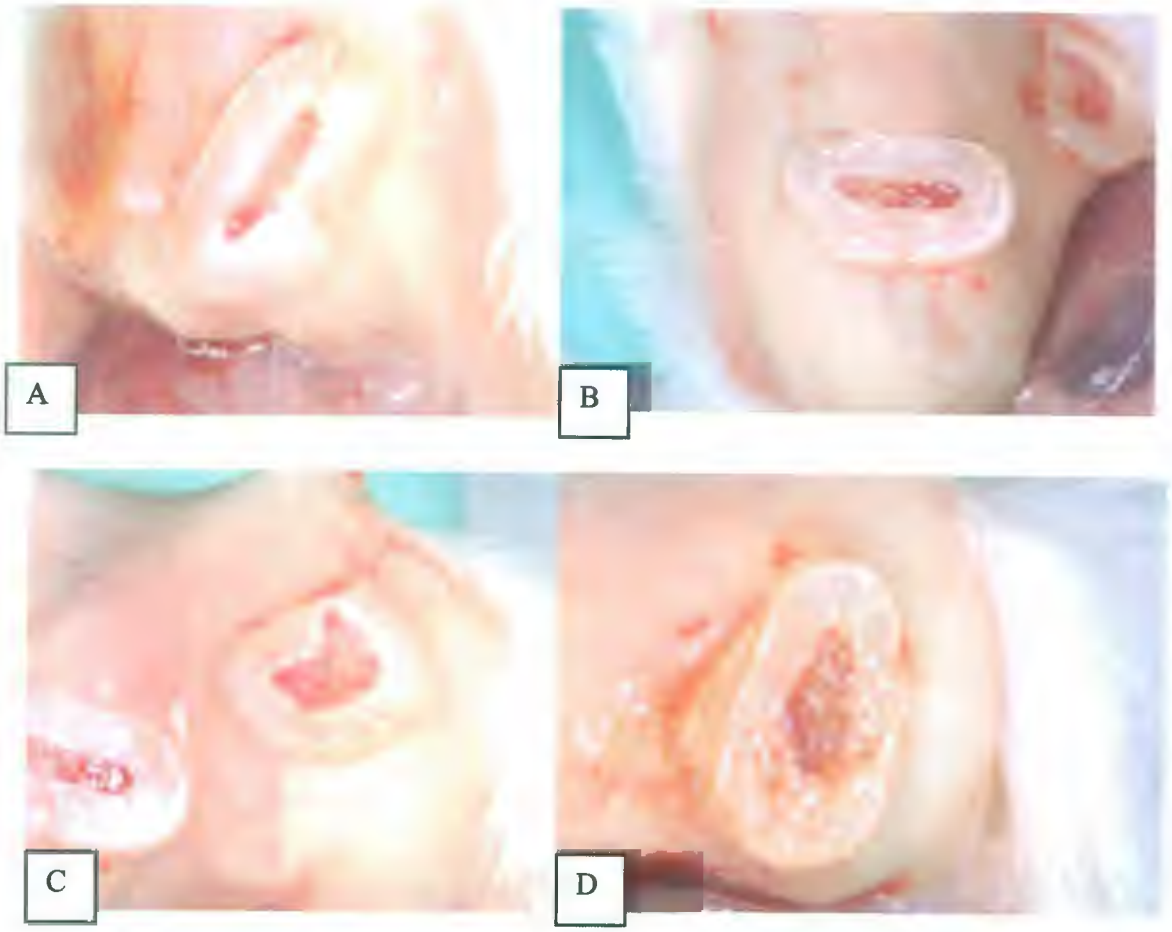

Figure 12: Implants after three weeks. (A) Empty Control; (B) DPF; (C) DPSC + DPF and (D) DPSC.

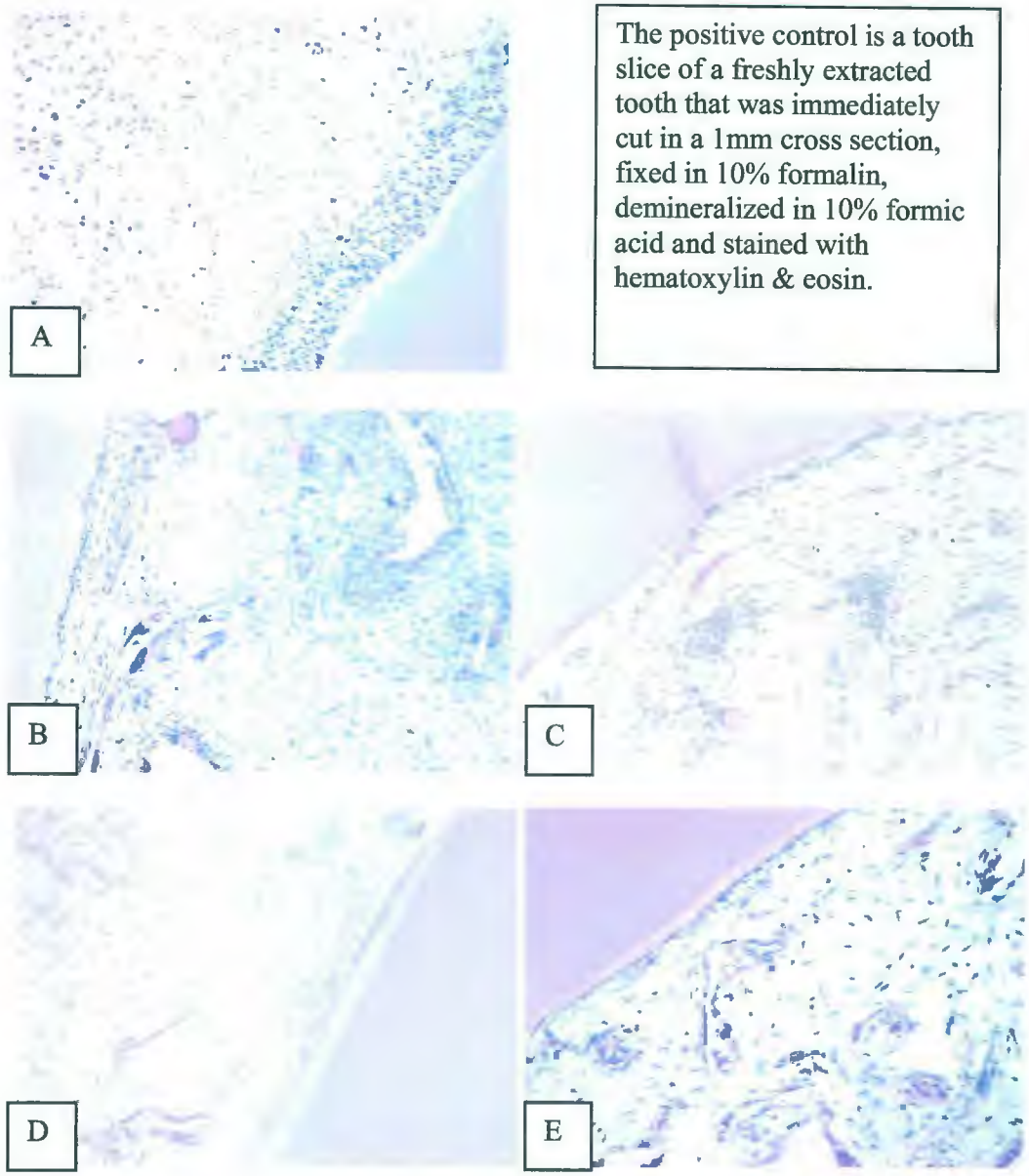

Figure 13: Hematoxylin & Eosin staining of engineered dental pulps (200X). (A) Positive control with a regular dental pulp, (B) engineered tissue using DPF, (C) engineered tissue from the empty control sample, (D) engineered tissue with DPSC, (E) engineered tissue with DPSC + DPF.

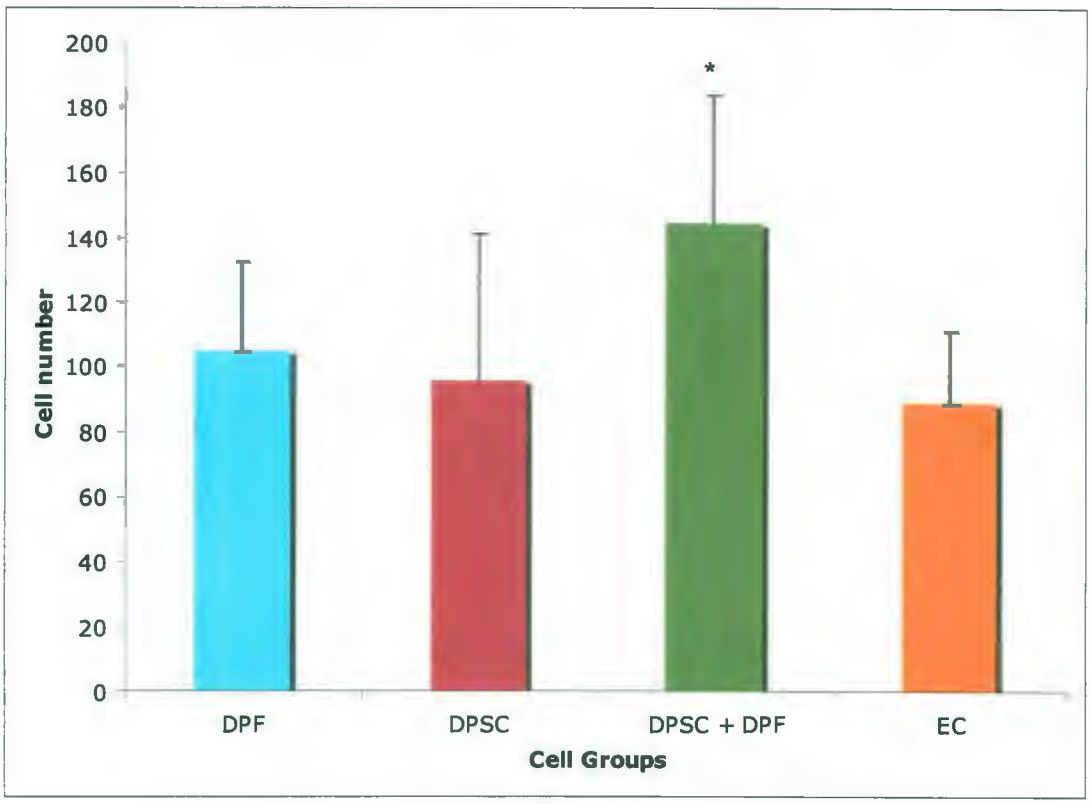

Figure 14: Number of cells lining the pre-dentin according to experimental conditions after 21 days implantation in the dorsal subcutaneous if SCID mice. (*, $p < 0.05$). (Total n=16).

Microvessel Density of tissues formed

Microvessel density was assessed after immunohistochemistry with Factor VIII.

We observed that the microvessel density is significantly higher in the groups DPSC + DPF and DPSC only, as compared to DPF only, or the empty scaffold controls (Figures 15 and 16).

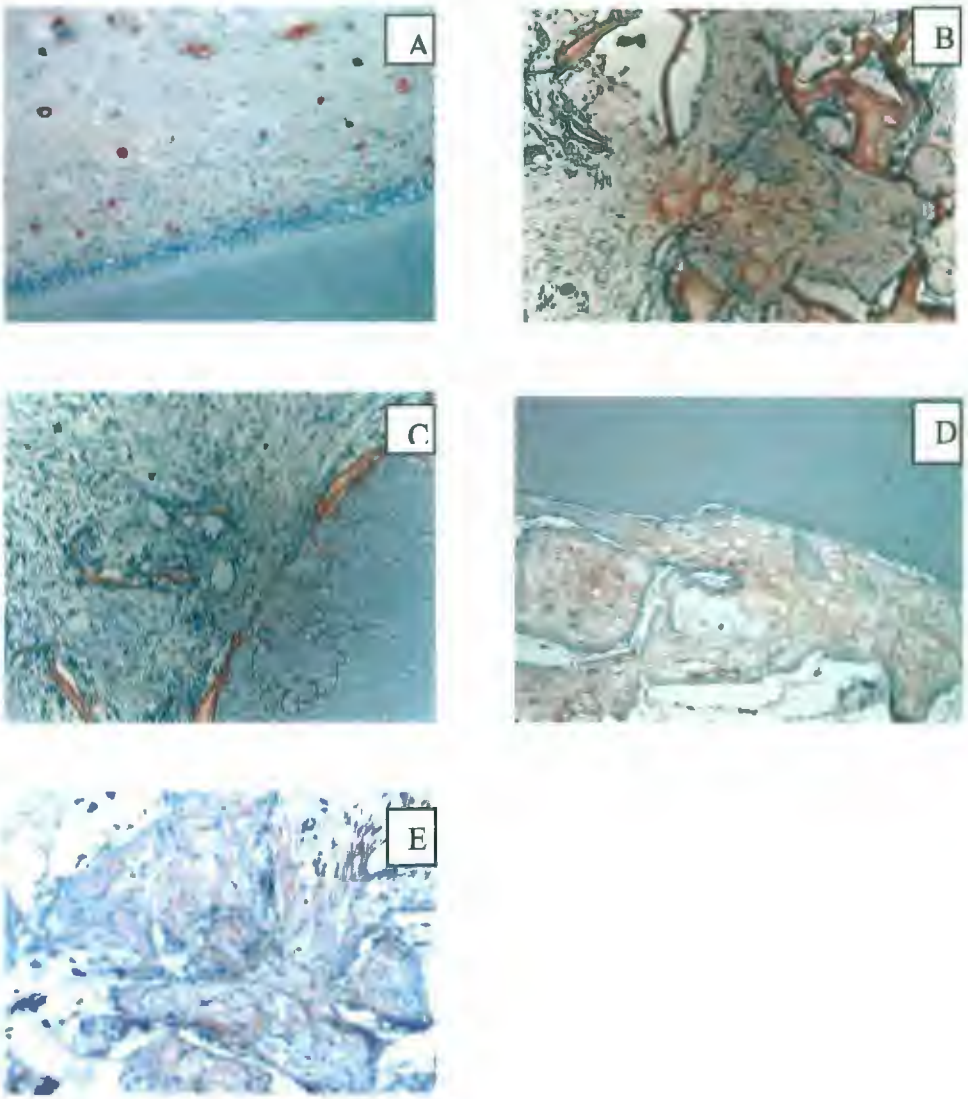

Figure 15: Factor VIII immunohistochemistry: (A) Positive Control: human pulp tissue; (B) Engineered tissue with DPSC + DPF; (C) Empty Control (implanted without seeded cells); (D) Engineered tissue with DPSC only and (E) Engineered tissue with DPF, 200X.

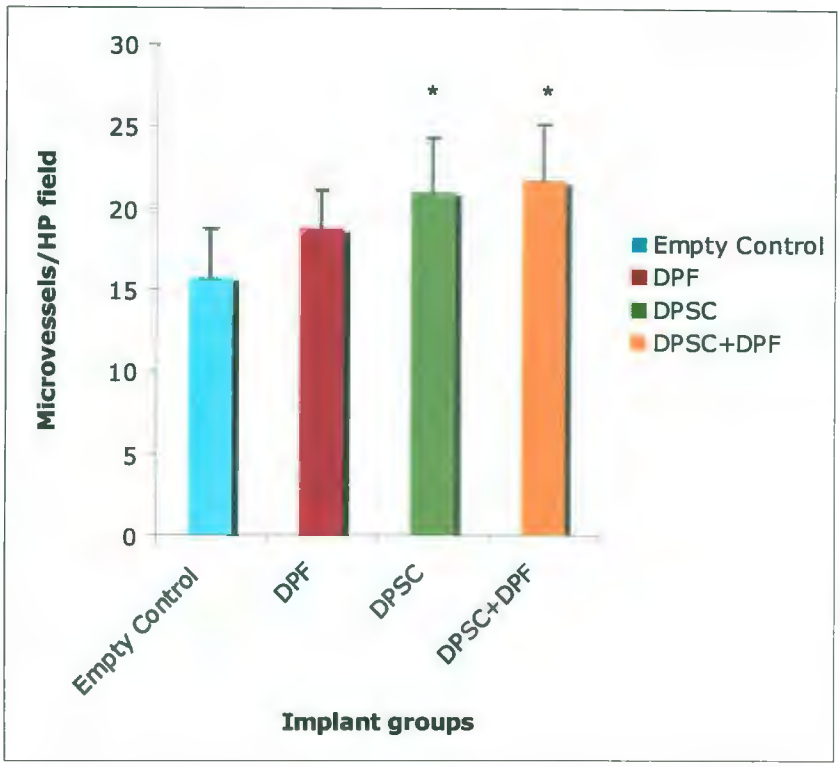

Figure 16: Microvessel density in engineered dental pulps, according to experimental conditions.(*, $p < 0.05$). (Total n=16).

5. Discussion

In this study we attempted to begin the characterization of engineered dental pulp tissues. In our *in vitro* experiments we studied the spatial distribution of cells (i.e. DPSC and DPF) in the scaffolds. Cell density and its spatial distribution inside the tooth-scaffold devices were evaluated over seven days.

A significant *in vitro* finding is revealed by cellular behavior from single cell population cultures to the co-culture situation, where we only observe cytoplasmic processes when we have both cells interacting together (total n=16). DPF cells are responsible for secreting collagen as an extra cellular matrix for protein synthesis and a sequence of morphogens such as fibroblast growth factor (FGF), and VEGF and others (Tran-Hung *et al.*, 2006) that are relevant for tooth morphogenesis, signaling to the DPSC tissue specificity related to the dental pulp. Stem cell mediated tissue engineering is maximized by tissue specific cells, producing a situation similar to what is found in natural tissues where stem cells interact with several other cell types in the tissues where repair and regeneration are occurring (Wagers and Weissman, 2004).

The *in vivo* results are encouraging to show that the principle of pulp cell therapy in a scaffold inside a tooth slice was feasible to generate tissue in the animal model, and the tissue morphology evaluated in this study resembles a dental pulp. These results corroborate previous data from our laboratory, when dental pulp stem cells from primary teeth (SHED) were transplanted into SCID mice in tooth slice/scaffold devices (Cordeiro *et al.*, submitted).

The findings related to microvascular network formation being significantly higher in the co-culture are consistent with data reporting that DPSC population was found intimately associated with blood vessels in regular pulp tissues through the identification of several markers of the microvascular network (Shi and Gronthos, 2003). This can mean that the tissues formed with tooth slice/scaffold devices seeded with DPSC and DPF resemble more what is found in regular pulp tissues.

Before one can attempt to apply these principles into clinical practice, many questions will need to be addressed. How to obtain enough vascular supply is vital to maintain an engineered tissue. As collagen type 1 is an important component of dentinal-pulp formation (Priam *et al.*, 2005) (Sumita *et al.*, 2006), it would probably be the most suitable scaffold for this clinical scenario.

Platelet-Rich Plasma (PRP) obtained from the patients own blood could be a vehicle to provide cell attachment into the scaffold, to be carried into the tooth and remaining pulp chamber. PRP has been reported to promote bone regeneration and also play a role in suppressing inflammation and stimulating tissue regeneration (El-Sharkawy *et al.*, 2007). Several growth-factors active in PRP play a key role in tooth morphogenesis: platelet-derived growth factors (PDGF-AB and PDGF-BB), transforming growth-factor-ß-1, insulin-like growth-factor-I, fibroblast-growth-factor (FGF-b), VEGF and others (El-Sharkawy *et al.*, 2007). Platelet-Rich Plasma has been proven to be an excellent graft carrier in bone regeneration procedures, and its gel form at room temperature would facilitate cell adhesion into the scaffold. Clinical case reports using bone marrow stem cells and platelet growth factor show, through computerized tomography, well integrated scaffolds adapted to the cortical bone (Filho Cerruti *et al.*, 2007).

Some confounding factors related to the regeneration ability of the patients are bacteriological invasion into the remaining pulp chamber and immune response, affected by the patient's systemic condition and age. Acceptance of cell therapy in this kind of self-contained environment- a tooth cavity with dentinal walls that would not allow inflammatory edema to occur, would be dictated by an inflammatory response from the host that would be tolerable. Also, occlusal load should be considered carefully, as it could mechanically stimulate or affect negatively the regeneration process. Treatment of the remaining dentinal walls with 10% EDTA without harming the remaining pulp tissue could be useful to liberate growth factors involved in cell signaling, using autogenous dentin induction to help in the process (Graham, Nör, Smith, *et al.*, 2006).

With further studies to better understand and orchestrate triggering factors and key elements that would stimulate the pulp regenerative process, novel approaches involving cell therapy inside a biodegradable scaffold might become an option to offer young patients who experienced trauma situations with fractured crowns of permanent teeth. This therapy would allow for the completion of root formation and therefore contribute to the longevity of the tooth (Nör, 2006). Situations where a direct pulp capping is required where the degree of pulp inflammation is still reversible, regeneration techniques for pulp tissue through cell therapy may become a feasible option to preserve pulp vitality and help stop the destructive process of the tooth organ.

Nevertheless, the principle of engineering a tissue with cellularity similar to the dental pulp inside the pulp chamber space of sliced teeth has been proven possible in the SCID mice model. The *in vitro* findings of cellular disposition with cytoplasmic processes

extending inside the dentinal tubules are encouraging to suggest that odontoblastic differentiation might be occurring.

6. Conclusions

Within the limitations of this study, we conclude that:

1. The visualization of DPSC and DPF with long term cell labeling through confocal laser microscopy seemed adequate to assess cellular density and spatial distribution of cells within tooth slices.

2. Cytoplasmic processes inside dentinal tubules observed in the co-culture samples after 7 days *in vitro* are suggestive of the presence of odontoblast like cells in the engineered dental pulp.

3. Co-implantation of DPSC and DPF seeded in tooth slice/scaffold devices into the subcutaneous of immunodefficient mice allowed for the engineering of a dental pulp-like tissue.

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Appendix

Laboratory Protocols

A. Cell Culture:

Human Dental Pulp Stem cells

Medium: 75ml flasks

- 500ml of low glucose Dulbecco's Modified Eagle Medium (DMEM)+
- 50ml (10%) Fetal Bovine Serum (Gibco),
- 5.5ml (1%) of Penicillin/Streptomycin

Freezing Medium:

- 45ml of FBS (90%)
- 5ml of DMSO (10%)

Keep in 4 Falcon flasks: ~12ml each

Cells are kept in a humidified CO_2 incubator at 37 ^{0} C. Medium was changed every other day.

When cells reach about 70% of confluency (after 2 to 3 days), they are detached from the 75ml flasks with:

1. 4ml Trypsin wash and quick shake in flask, aspirate right away.
2. 2ml Trypsin for 3 min in incubator, gently tapping the flask to help detach the cells.
3. 4ml of medium to neutralize trypsin, transport the cells to a falcon tube.
4. Centrifuge in program 1 for 5 min.
5. Aspirate liquid from cell pellet.
6. Resuspend cells in medium: 1ml per flask (usually pass at a proportion of 1:3 flasks).

7. New flasks are filled with 9ml of medium when cells are transported to it.
8. Change medium the next day to eliminate trypsin toxicity from medium.

To freeze, centrifuge and resuspend with freezing media: 1ml per vial tube.

Human Dental Pulp Fibroblasts

Medium: 75ml flasks

- 500ml of high glucose Dulbecco's Modified Eagle Medium (DMEM)+
- 50ml (10%) Fetal Bovine Serum (Gibco),
- 2.75ml of Penicillin/Streptomycin
- 5.0 ml of L-Glutamine

Freezing Medium:

- DMSO: 5ml
- Medium: 40ml
- FBS: 5ml

B. Scaffold Fabrication Inside the Teeth Slices:

The technique was developed to prepare polymer scaffolds inside the canal of the tooth slice is described below:

5% Poly-L-lactic acid (PLLA) scaffolds were prepared inside the canal space of a sliced tooth. The description of this protocol is in the appendix section

Materials:

NaCl (sieved 250μm, Sigma)

5% Poly-L-lactic acid (PLLA, Resomer®, BOEHRINGOR INGELHEIM)

Chloroform by J T Baker, in 100ml glass beakers.

Parafilm®, by Pechiney Plastic Packaging (Menasha, WI)

Methods:

Day 1: prepare a 5(w/v) % solution of PLLA of PLLA in chloroform: 1g PLLA in 20ml chloroform, using glass pipettes. Leave it covered with poked parafilm overnight to evaporate the chloroform.

Day 2: Teeth slices are dried in sterile gauze, placed in a glass slab and NaCl was sprinkled inside the canal area. Drops of PLLA are applied on top of the NaCl, letting it soak in and fill the canal space. Excess material is cleaned off the dentin area. The procedure is repeated on the other side of the slice, trimming excess material, sprinkling NaCl and PLLA until the canal space is completely filled with the materials. The samples are placed inside the beakers to allow the setting of the polymer overnight, covered with poked parafilm.

Day 3: Wash the samples with PBS-1X to solubilize the NaCl for two days, changing PBS-1X four times a day.

Samples are maintained in PBS-1X at 4^0 C, in 24 well plates.

Prior to using the samples for experiments, a final check on excess material outside of the canal area of the tooth slices is performed with a scalpel # 15 and sterile gauze, returning the samples to the wells with PBS-1X at 4^0 C.

Day 1: Three-dimensional samples of tooth slice/scaffolds are prepared and sanitized in 100% ethanol for 10 min, 90% ethanol for 10mim, 80% ethanol for 10 min, 70 % ethanol for 10 min, sterile PBS-1X for 1 hour, changed PBS-1X to spend overnight at 4^0 C.

Day 2: The samples are treated with 10% EDTA for one hour, then receive three washes with fresh sterile PBS-1X for 30 min each, and are ready for cell seeding procedures.

C. Cell density inside the tooth slice devices:

Cell seeding quantity was determined with the following calculations:

Maxillary molars (n=10):

$V= 0.77 \times 6.27 \times 1.4 = 6.76\ mm^3$

$A= 29.38\ mm^3$

Mandibular molars (n=10):

$V= 0.71 \times 4.52 \times 3.57 = 11.46\ mm^3$

$A= 43.76\ mm^3$

Scaffold: (as standard)

$V= 1 \times 6 \times 6 = 36\ mm^3$

$A= 96\ mm^3$

$V = h \times w \times d$ $A= 2wd + 2dh + 2hw$

As we know we can seed in the standard scaffold (6x6x1): $1x10_6$, we determined that:

Small pulp chambers: we seed $3.3 \times 10_5$

Large pulp chambers: we seed $5 \times 10_5$

D. Seeding cells in Matrigel® inside the tooth-scaffold devices

1. Preparing cells to be seeded

Materials:

- DPSC and DPF (primary cell lines, passages 5-8) in 150ml flasks
- DMEM low glucose and high glucose media, respectively, at 37°C
- Trypsin, 37°C
- Trypan Blue Stain 0.4%

Methods:

- Trypsin wash the cells: 8ml
- Incubate cells in 4ml of Trypsin for 3-5 min at 37°C
- Neutralize Trypsin with 8ml of media
- Collect 12ml to be centrifuged for 5 min, 800 rpm (program 1)
- Aspirate extra media, leave cell pellet in the bottom
- Resuspend cells in 4ml media
- Obtain 50µl of resuspended cells + 50µl of Trypand Blue
- Count cells with hemocytometer: A+B+C+D/4= X x 10^4 per ml (x 4 ml of resuspension.

2. Seeding cells into the teeth-scaffolds devices

Materials:

- Vials with cell pellets, on ice
- Matrigel®, on ice
- PLLA scaffolds, previously sanitized in ethanol grades, washed 3X in 1X-PBS

Methods:

- After counting, distribute resuspended cells in aliquots of the desired quantity of 5cells in individual vials ($5x10^5$ per tooth/scaffold sample)
- New centrifuge of 1000 rpm for 1 minute to obtain a cell pellet
- Aspirate excess media and leave 9µl of media with pellet
- Add 9µl of Matrigel® to the mix, take it to the scaffold
- Leave in incubator for 30min.
- Transport samples in well plates on ice to be implanted subcutaneously in SCID mice, two samples per mice.

E. In Vitro Experiment Design:

Cut teeth slices.

Prepare samples of teeth-scaffold for each cell group: DSPC, DPF, SC+F, Empty Control

Sanitize them in alcohol grades from 100%, 90%, 80% and 70% for 10 min each, one hour of PBS wash, fresh PBS overnight.

Next day, treat them with EDTA 10% for one hour prior to seeding, 1X- PBS washes three times, each 30 min.

The seeding of the cells in scaffold will happen on the "base line" day and I distribute evenly the same amount of cells in different vials.

1. Stain both population of cells with respective probes in PBS for 40 min, obtain pellet
2. Wash pellets twice in warm media for 30 min (first 30 min interval in incubator)
3. Seed cells in tooth/scaffold sample in Matrigel®.
4. Incubate for one hour.
5. Add media to the samples.
6. Labtech® 2 well covered chambers will hold the samples for each cell group.
7. Cover the samples with foil, change media every other day.

To ensure quality of pictures I treat them with HBSS twice for 30 min before acquiring pictures. Samples are discarded after image capture: three fields on each sample at 400X.

<u>To measure:</u>

Quantification of cells overtime

Cellular disposition on tooth/scaffold structure

F. Methodology for Long Term Cell Labeling Probes

A protocol was developed and optimized to determine a concentration of the fluorescent dyes that can be used without being harmful and toxic to cell survival.

After determining a working concentration of 10μM for CFDA SE (green dye) and 1μM for SNARF®1 (red probe), experiments were performed to evaluate cell survival over a period of five days, where images are captured at days zero, one, three and five.

The samples are previously treated with 2 washes of 30 minutes of HBSS (Hank's Balanced Salt Solution) without Magnesium and Calcium in the humidified CO_2 incubator at 37 ^{0} C, before undergoing UV light in inverted microscopy for image capturing.

At seven days, with inverted confocal laser microscopy (Olympus), images were obtained of the samples with co-culture of DPSC and DPF, as well as samples that had single cell populations with each cell type, and empty controls without any cells in it.

1. Carboxy Fluorescein Succinimidyl Ester (CFDA SE®) green fluorophore

Molecular Probes give you a wide range of working concentration of the dye for you to try in the specific cells you are working with: 0.5-25μM

MW= 557.47

Prepare a 10mM CFDA SE stock solution immediately prior to use by dissolving 500μg (quantity that comes in vial) in 90 μl of anhydrous DMSO provided in the kit.

For a concentration of 25μM I make the calculation: 10mM=10,000μM divided by 25μM, gives me a proportion for 1/400μM.

So, for each 399µl of PBS: 1µl of CFDA SE stock solution – for [25]µM.

2. SNARF®-1 carboxylic acid, acetate, succinimidyl ester red fluorophore

Carboxy SNARF®-1 is a long-wavelength fluorescent pH indicator developed by Molecular Probes. It undergoes a pH-dependent wavelength shift, thus allowing the ratio of the fluorescence intensities from the dye at two emission wavelengths to be used for more accurate determinations of pH. Excitation of the dye happens between 488nm and 530nm, while fluorescence emission happens at two wavelengths, typically about 580nm and 640nm.

Similarly, the adequate working concentration was found to be 1µM to be used with DPF. The protocol follows:

Molecular Probes give the following information:

SNARF®-1 MW: 592.56

Concentration range given by Molecular Probes:1-20µM

1 vial comes with 50µg

$50\mu g/10_6$ divided by 592.56=Mole/10_6=0.0844

M= 1 mole/1L= $0.0844/10_6$ divided by $1x10_3x10_3 = 0.0844\mu M$

The concentration ranges from 1-20µM:

Stock: $\frac{20\mu M}{1 L} = \frac{50\mu g/592.56}{V}$

$V = \frac{50/592.56}{20/10_6}$

V=4,2189ml

For 20µM= 4.2189 ml DMSO

50μM of stock solution in 843.78 μl of DMSO

For a volume of 1 ml, a concentration of [1μM], I dilute100μl stock + 990μl of PBS= 1ml

G. Staining DPSC with Carboxyfluorescein Succinimidyl Ester (CFDA SE®) into the teeth-scaffold devices

1. Preparing dental pulp stem cells to be stained

Materials:

Sterile 1X- PBS

Stock of 10μM of CFDASE: in vial provided in the kit dilute 90μl of anhydrous DMSO

Cell media for DPSC, low glucose DMEM

Vials with cells, on ice

Methods:

- Centrifuge cells and obtain pellet, aspirating supernatant.
- Concentration of 10μM of CFDASE is required to stain DPSC
- In falcon tube dilute 10μl of CFDASE in 10 ml of PBS-1X to obtain the working concentration of 10 μM
- Resuspend cell pellet in the PBS containing the dye
- Place in incubator for 30-40 min for 37°C
- Re-pellet the cells by centrifugation and resuspend in fresh pre-warmed medium.
- Incubate for another 30 minutes to ensure complete modification of the probe.
- Wash the cells again in fresh media, 2X
- Re-pellet to seed cells in scaffolds with Matrigel®

2. Seeding cells in Matrigel® inside the tooth-scaffold devices

The cells were centrifuged again to obtain a pellet to be mixed with Matrigel® and cell culture medium in a 1:1. The mix is applied and absorbed by the scaffold inside the teeth. The samples are placed inside Labtech® covered chambers inside the incubator at 37 ^{0} C for one hour.

Finally, Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA), supplemented with 10% Fetal Bovine Serum (Gibco), 125 units/ml Penicillin, and 125 ug/ml Streptomycin are dispensed in the covered chambers where the samples are, maintained in a humidified CO_2 incubator at 37 ^{0} C. Medium was changed every 2 days.

The samples are covered with aluminum foil because the fluorophore is light sensitive.

H. SNARF®1 cell labeling for DPF and Seeding in Matrigel® into Tooth-Scaffold devices

1. Preparing dental pulp fibroblasts to be stained

Materials:

- DPF (primary cell lines, passages 5-8) in 150ml flasks
- DMEM High Glucose media at 37°C
- Trypsin, 37°C
- Trypan Blue

Methods:

- Trypsin wash the cells: 8ml
- Incubate cells in 4ml of Trypsin for 3-5 min at 37°C
- Neutralize Trypsin with 8ml of media
- Collect 12ml to be centrifuged for 5 min, 800 rpm (program 1)
- Aspirate extra media, leave cell pellet in the bottom
- Resuspend cells in 4ml media
- Obtain 50µl of resuspended cells + 50µl of Trypan Blue
- Count cells with hemocytometer: A+B+C+D/4= X x 10_4 per ml (x 4 ml of resuspension

2. Staining with SNARF ®1 Cell Labelling

- Centrifuge cells and obtain pellet, aspirating supernatant.
- Concentration of 1µM of SNARF 1 is required to stain cells safely

- In stock vial of 50 mg of SNARF 1 dilute 843.78µl of anhydrous DMSO (calculations according with MW)
- For 1 ml of pre-warmed PBS-1X add 2 µl of stock solution of SNARF 1 to get 1µM concentration of the product.
- Place in incubator for 40 min for 37°C
- Re-pellet the cells by centrifugation and resuspend in fresh pre-warmed medium.
- Incubate for another 30 minutes to ensure complete modification of the probe.
- Wash the cells again in fresh media
- Re-pellet to seed cells in scaffolds with Matrigel®

3. Seeding DPF cells into the teeth-scaffolds devices

Materials:

- Vials with cell pellets, on ice
- Matrigel®, in ice
- PLLA scaffolds, previously sanitized in ethanol grades, washed 3X in 1X-PBS

Methods:

- After counting, distribute resuspended cells in aliquotes of the desired quantity of cells in individual vials ($5x10^5$ per tooth-scaffold sample)
- New centrifuge of 1000 rpm for 1 minute to obtain a cell pellet
- Aspirate excess media and leave 9µl with pellet
- Add 9µl of Matrigel® to the mix, take it to the scaffold
- Leave in incubator for 60min Add media to the well
- Labtech® 2 well covered chambers will hold the samples for each cell group

- Cover the samples with foil, change media every other day

The samples that have both cells in the teeth-scaffold system were stained first and then joined at the time of seeding with Matrigel®.

The seeding of the cells in scaffold will happen on the "base line" day and I distribute evenly the same amount of cells in 7 different vials.

1. *Stain cells only with [1μM] of SNARF® 1 in PBS for 40 min, obtain pellet*
2. *Wash pellets twice in warm media for 30 min (first 30 min interval in incubator)*
3. *Seed cells in tooth/scaffold sample in Matrigel®*
4. *Incubate for one hour*
5. *Add media to the samples*
6. *2 well covered chambers by Labteck® plates will hold the samples for each cell group*
7. *Cover the samples with foil, change media every other day*

I. DSP immunostaining Protocol:

Deparafination:

1. Xylene 3x for 5 min
2. 100% Ethanol 3x 1 min
3. 90% Ethanol 5 min
4. 80% Ethanol for 5 min
5. 70% Ethanol for 5 min
6. Rinse in tap water for 5 min

Endogenous Peroxidase Blockage

1. Incubate sections for 30 minutes in 3% H2O2 in methanol, exchanging the liquid 15 min into it to improve efficacy of peroxidase blockcage.
2. Wash in buffer twice for 5 min, 2X

Antigen Retrieval:

1. Place the samples in flat surface
2. Add 1 drop of Trypsin solution A over each slice, 1 drop pf Solution B and one more drop of Solution A.
3. Place slides in humidified incubator at 37°C for 60min
4. Wash in buffer for 5 min

Background blockage

1. Incubate sections for 20 minutes with diluted normal horse serum
2. Blot excess serum from sections

Antibody Reactions:

1. Apply primary antibody DSP diluted 1:500 in buffer in sections, leave them covered overnight at 4°C, then incubate it at 37°C for 30 min.
2. Wash slides 5 min in buffer
3. Incubate sections for 30 min with diluted biotinylated "universal" secondary antibody
4. Wash slides twice for 5 min in buffer
5. Incubate sections for 30 min with VECTASTAIN®R.T.U. ABC Reagent.
6. Wash slides for 5 min in buffer
7. Prepare DAB peroxidase solution by adding to 2.5ml of distilled water one drop of hydrogen peroxide, one or two drops of substrate reagent (brown stain) and one drop of buffer from the DAB kit.
8. Incubate sections in peroxidase substrate solution until desired stain intensity develops (2-10 min). Watch the reaction in microscope and when desired stain is obtained, stop the reaction with distilled water.
9. Counterstain slides with Hematoxilyn
10. Rinse sections in tap water.
11. Mount with coverslides in aqueous mounting media.
12. Seal with nailwear.