ODONTOGENIC CELL CULTURE IN PEGDA HYDROGEL SCAFFOLDS FOR USE IN TOOTH REGENERATION PROTOCOLS

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ABSTRACT

In order to obtain a tooth-like structure, embryonic oral ectoderm cells (EOE) and bone marrow-derived stem cells (BMSC) were stratified within a synthetic hydrogel matrix (PEGDA) and implanted in the ileal mesentery of adult male Lewis rats. Whole-mount in situ hybridization was used to evaluate the expression of Pitx2, Shh and Wnt10a signals indicative of tooth initiation. In rats, expression of the three markers was present in the oral ectoderm starting at embryonic stage E12.5, which was therefore selected for cell harvesting. Embryos were obtained by controlled service of young female Lewis rats in which estrus was detected by impedance reading. At E12.5, pregnant rats were humanely euthanized and embryos were collected. The mandibular segment of the first branchial arch was dissected and the mesenchyme separated from the ectoderm by enzymatic digestion with pancreatin trypsin solution. BMSCs were collected by flushing the marrow of tibiae and femurs of adult Lewis rats with α-MEM and cultured in α-MEM in 25 cm² flasks. Second passage BMSC's were recombined with competent oral ectoderm (E12.5-E13) stratifying them within a 3D PEGDA scaffold polymerized by exposure to UV (365nm) inside a pyramidal polypropylene mold. Constructs were incubated from 24 to 48 hrs in α-MEM and then implanted for four to six weeks in the mesentery of adult male (3-6 month old) Lewis rats. 76 constructs were implanted (37 experimental, 27 negative controls and 12 positive controls). Upon maturation, constructs were harvested, fixed in buffered formalin, processed and stained with hematoxylin eosin (HE). Histological evaluation of the experimental and negative constructs showed that BMSCs underwent an apoptotic process due to lack of matrix interactions, known as anoikis, and were thus incapable of interacting with the competent ectoderm. In contrast, embryonic oral ectoderm was able to proliferate during the mesenteric implantation. In conclusion, PEGDA scaffolds are incompatible with BMSCs, therefore it is essential to continue the search for an ideal scaffold that allows proper tissue interactions.

Keywords: *Polyethyleneglycol diacrylate - 3D scaffolds - embryonic structures - stem cells - mesentery.*

CULTIVO DE CÉLULAS ODONTOGÉNICAS EN SOPORTES DE HIDROGEL PEGDA PARA USO EN REGENERACIÓN DENTAL

RESUMEN

Para lograr la formación de una estructura similar a un diente se estratificaron células de ectodermo oral embrionario (EOE) con células troncales de medula ósea (BMSC) dentro de una matriz de diacrilato de polietilenglicol (PEGDA) que se implantó en el mesenterio ileal de machos adultos de ratas Lewis. Mediante hibridización in situ de bloque completo se evaluó la expresión de tres genes iniciadores putativos de la formación dental (Pitx2, Shh y Wnt10a), estableciendo que en ratas las señales iniciadoras de dentogénesis aparecen entre E12.5 y E13. El tejido ectodérmico embrionario se obtuvo haciendo cruces controlados de hembras a las que se les detectó el estro mediante impedanciometría. En E12.5 las hembras se sacrificaron y se extrajeron los embriones. Se disecó la porción mandibular del primer arco branquial y el ectodermo se separó del mesénquima mediante disociación enzimática con una solución de pancreatina tripsina. Las BMSC se extrajeron de los huesos largos de las extremidades inferiores de ratas mediante lavado con α-MEM y se cultivaron en cajas de 25cm² hasta un segundo pasaje. Las BMSC fueron recombinadas con ectodermo embrionario competente (E12.5 - E13) estratificándolas en un soporte tridimensional de PEGDA, polimerizado con luz ultravioleta (365nm) dentro de un molde piramidal de polipropileno (PP). Los constructos se cultivaron entre 48 y 72 horas en α-MEM y posteriormente fueron implantados en el mesenterio ileal de machos adultos (3 a 6 meses de edad) por un período de cuatro a seis semanas. Se implantaron 76 constructos (37 experimentales, 27 controles negativos y 12 controles positivos). En la fecha determinada los animales se sacrificaron mediante asfixia con una mezcla de CO_2 y aire recuperando los constructos que se fijaron en formalina tamponada para luego procesarlos y teñirlos con hematoxilina eosina (HE). La evaluación histológica de los constructos experimentales, positivos y negativos mostró que las BMSC incluidas en el hidrogel sufrieron un proceso de apoptosis conocido como anoikis que impidió su interacción con las células ectodérmicas. En contraste el EOE proliferó durante el período de implantación. A futuro se debe buscar la matriz portadora ideal que permita el confinamiento de los dos grupos celulares y que brinde el soporte estructural necesario para la proliferación de las BMSC facilitando su interacción con las células inductoras de origen ectodérmico.

Palabras clave: *Diacrilato de Polietilenglicol (PEGDA) - soporte tridimensional - estructuras embrionarias - células madre - mesenterio.*

INTRODUCTION

The need to restore or replace deteriorated or lost organs as a result of alterations in their formation, pathology, trauma or any other event affecting their integrity has driven the search for rehabilitating therapies. Some of these include repair or replacement by means of autologous transplants, allogeneic transplants and more recently, tissue engineering, which integrates cell biology, molecular biology, genetics and biotechnology to produce complex tissues or organs. This study seeks to apply some of the basic principles of tissue engineering to obtain a complex structure formed by tooth-like tissues and use it in tooth regeneration trials.

Tooth formation is the result of sequential, reiterative, and reciprocal interactions between ectodermal and mesenchymal tissues, through the expression of signaling molecules families driving tissues and the cells contained within along diverse differentiation pathways[1]. A wide range of studies at cellular and molecular levels have provided sufficient evidence to establish that the embryonic oral epithelium provides inductive signaling for tooth initiation. In the early stages of mandibular development in mice, all the ectomesenchymal cells seem to be capable of responding to signals from the oral epithelium[2]. The ability of the embryonic oral epithelium in early developmental stages (competent dental epithelium) to direct odontogenesis when recombined with non-oral ectomesenchyme suggests that ectomesenchymal cells are plastic in their responses to oral epithelial signals [3]. The use of embryonic tissues as source of inductive signals for organogenesis and their subsequent transplantation to receptor sites in adult individuals for the development of a complex multicellular structure *in vivo*, requires an animal model in which immunity due to histocompatibility does not pose another variable, therefore it is mandatory to use an inbred strain for this type of experiment. The Lewis inbred rat strain is one of the most frequently used animal models for transplant experiments[4] due to its high level of homozygosity.

Cellular proliferation and maturation during organogenesis requires a support structure in order to facilitate interactions, and in this regard the function of the scaffold has evolved into an instructive microenvironment facilitating cell adhesion, migration, differentiation and subsequently its own degradation[5]. Synthetic polyethylene glycol diacrylate (PEGDA) scaffolds are commonly used biomaterials in tissue engineering because their chemical and physical properties such as resistance to flexion, tension, compression and shearing can easily be modified according to the individual needs of the developing tissue[5,6]. They are used as drug and cell transporters[7], have been approved by the FDA (US Food and Drug Administration) for various medical applications[6] and some are formed from a variety of polymers that are water-soluble or interact with water, endowing them with high permeability[8]. They can be molded by photopolymerization processes gentle enough to be performed in presence of living cells, which in turn can become homogeneously distributed within the structure[9]. Polyethylene glycol based hydrogels have proven to be biocompatible, and support the viability of cells encapsulated within them [10,11]. Implantation of PEGDA constructs has shown that the scaffold breaks down, allowing the formation of a cell matrix with the microscopic structures distinctive of the different cell layers included in it[12]. The aim of this study was to combine rat embryonic oral ectodermal cells (with potential to induce odontogenesis) and mesenchymal stem cells from adult rats in a hydrogel scaffold and implant them *in vivo* to achieve the formation of a structure containing tissues of dental origin.

MATERIAL AND METHODS

Animals

This study followed the legal provisions of The International Guiding Principles for Biomedical Research Involving Animals developed by the CIOMS (Council for International Organizations of Medical Sciences), which includes ethical and legal aspects of experimenting with animal models. The laboratory procedures were performed according to the standards for Biosafety for managing and disposing of biological specimens of the Dental Research Center of the School of Dentistry, Pontificia Universidad Javeriana and the Center for Animal Experiments of the School of Dentistry, Pontificia Universidad Javeriana. This Project was approved by the Ethics and Research Committee of the School of Dentistry, Pontificia Universidad Javeriana as part of the project "*In vivo* Obtention of a tooth-like structure from odontogenic rat cells in a hydrogel scaffold".

Approximately ten 8-week-old sibling pairs of male (150-225g) and female (100-200g) Lewis

(LEW/ SsNHsd) rats were purchased from Harlan Laboratories, Inc. (Indianapolis, IN) and mated monogamously to generate the foundation colony. The pairs were retired from the foundation colony after the third pregnancy and replaced with a new sibling pair. The offspring produced were used for breeding either in the foundation colony or in the production colony used for experiments. Male and female rats in the production colony were group-housed in a One Cage® ventilated microisolator (Lab Products, Inc.) (four or five rats per cage) in disinfected polycarbonate cages (36 cm × 49 cm × 21.2 cm; One Cage, Lab Products, Inc., Seaford, DE) according to age and sex. For breeding, rats were housed in pairs in polycarbonate cages (36 cm × 23.5 cm × 21.5 cm; One Cage, Lab Products, Inc.). All cages were covered with filter tops and contained sterile pine wood chips (Depósito de Maderas las Palmas SAS, Bogotá, Colombia). The cubicle containing the microisolator had an average of 50 filtered air (HEPA)turnovers/hour, temperature 22+/- 1°C, relative humidity 55 to 65% and 12:12 light/dark cycle (lights on at 06:30 a.m. and off at 6:30 p.m.). All animals were transferred to clean, disinfected cages with a fresh bed twice a week. Sterilized feed (Rodentina Agrinal Colombia SA.) and water were provided *ad libitum* from the time of entry until euthanasia. Once a week, water was supplemented with a vitamin/amino-acid complex (Promocalier L, Laboratorios Calier SA.) to compensate for the nutrients lost through feed sterilization.

Microbiological and serological monitoring was performed twice a year. The results were negative for the following pathogens: *Mycoplasma pulmonis*, Sendai virus, CAR bacillus, *Encephalitozoon cuniculi*, IDIR, Hantaan, LCMV, MAD1, MAD2, RPV, RMV, KRV, H1, PVM, RCV/SDAV, REO3, RTV and *Clostridium piliforme*. The rats were also negative for *Helicobacter* spp., *Pasteurella spp*., *Streptobacillus moniliformis*, *Streptococcus (Streptoccoco β haemolitic* group A), *Salmonella spp*, *Leisteria monocytogenes*, *Corynebacterium kutscheri*, *Pneumococcis spp, Sarcoptes scabiei*, *Cestodes* (intestinal parasites), *Spironucleus muris* (intestinal parasites), *Giardia muris*, *Siphacia obvelata*, *Aspiculuris tetraptera*, all arthropods, all helminthes and all intestinal protozoa, therefore the health of the individuals housed at the SEA FOUJ qualified as SPF (Specific Pathogen Free).

Population

34 pregnant females (10 - 38 weeks old) were used as a source of embryonic tissues, 70 males (10 - 38 weeks old) as implant recipients and 5 females (10 - 21 weeks old) as bone marrow donors (109 animals altogether).

Humane euthanizing conditions

All individuals were euthanized by asphyxia with a mixture of CO_2/air followed by cervical dislocation.

Culture conditions

Whenever culture medium is mentioned, it refers to α-MEM (Gibco 12000-022) supplemented with 10% bovine fetal serum (Gibco 16000-044) and 1% penicillin /streptomycin /amphotericin B (Gibco 15240-062). All incubation steps were performed at 37ºC and 5% CO_2, and the culture medium was changed every two days. *In vitro* culture time for the constructs was 48 to 72 hours.

Bone marrow stem cell culture

The aseptic dissection technique for lower limb bones (tibia and femur) of humanely euthanized young females to obtain bone marrow stem cells was standardized. The marrow was flushed using a syringe with an 18-gauge needle loaded with 1 ml of culture medium, mechanically disaggregated using the same needles, and centrifuged at 2600xg for five minutes. The supernatant was discarded, the precipitate re-suspended in culture medium and seeded in 25 cm^2 cell culture dishes to establish the primary culture. When 80-90% cell confluence was reached, cells were detached by incubation with 1 ml trypsin-EDTA solution (Sigma T-4049), collected by centrifuging, and transferred to fresh vials at a density of 5-7 x 10^5 cells/vial, according to the Neubauer chamber count using the 0.4% Trypan Blue (Sigma T-8154) exclusion principle. Constructs were produced using the detached cells when the first passage was confluent (second passage).

Embryo production

In order to perform controlled crosses, estrus was determined in in the production colony females by measuring the vaginal wall impedance using an estrus cycle monitor (EC-40 Estrus Cycle Monitor®, Fine Science Tools). Females in estrus were mated during the night with trained males, separated the next day and the service was confirmed by evaluating the presence of a vaginal plug or sperm in vagi-

nal lavage, in which case the female was considered to be pregnant and day zero for embryo development was set.

The time to harvest embryos was determined by evaluating the concurrent expression of three putative genes, namely *Pitx2, Shh* and *Wnt10a*, with the whole-mount *in situ* hybridization technique at E11.5, E12, E12.5, E13 and E13.5 using RNA probes (sense and anti-sense). The probes were made from plasmids for each target gene donated by Dr. Irma Thesleff of the Institute of Biotechnology, University of Helsinki. Briefly, the eluted plasmid was amplified by cloning using an *E. coli* transforming strain, purified and linearized using restriction enzymes. The linearized plasmid was purified again, transcribed to obtain the RNA probes, which were applied to the embryos to evaluate the expression of the target genes.

On the day set for embryo harvesting, pregnant females were humanely euthanized and a midventral laparotomy was used to expose and remove the uterine horns which were placed in PBS with calcium and magnesium (DPBS) (Sigma D-1283). Embryos were removed from the yolk sac and placed in clean DPBS solution.

Embryonic ectoderm tissue preparation

Using hypodermic needles under a stereomicroscope (Nikon SMZ 1500), the mandibular segment of the first branchial arch was dissected and divided into two halves. The posterior segment of each of the two halves was again dissected, separating the portion containing the competent embryonic oral ectoderm (EOE) (Fig. 1 a and b). The dissected fragments were treated with 1 ml of trypsin-pancreatin solution (Sigma T-4799-Sigma P-3292) and incubated for 20 minutes at 37ºC to separate the ectodermal layer from the mesenchymal tissue (Fig. 1 c). After enzymatic digestion, the EOE fragments were incubated in culture medium at 37ºC until the three-dimensional constructs were prepared, procedure that was completed within the hour following euthanasia (Fig. 1 d).

Construct preparation

Three groups of constructs were prepared: experimental (seeded with BMSC y el EOE), negative control (seeded only with BMSC) and positive control (seeded with tooth germ ectodermal and mesenchymal cells at E12.5, E19 and 4PN).

The PEGDA was prepared at a concentration of 10% in PBS supplemented with a 1% antibiotic and antimycotic solution, to which a 0.1% solution of photoinitiator (Ciba, Irgacure 2959) was added. Polymerization was achieved by exposure to a 365nm UV radiation (Spectroline®BIB-150P lamp), in a polypropylene (PP) pyramidal mold maintained in an inert atmosphere (N_2 flow) at a distance of 15cm. Temperature of the mold was kept at 37ºC. Hydrogel loading capacity was tested by adding 200,000; 400,000 and 700,000 cells per construct, finding that full polymerization was achieved when 400,000 cells were included in 25 µl of total hydrogel volume. After polymerization the construct was

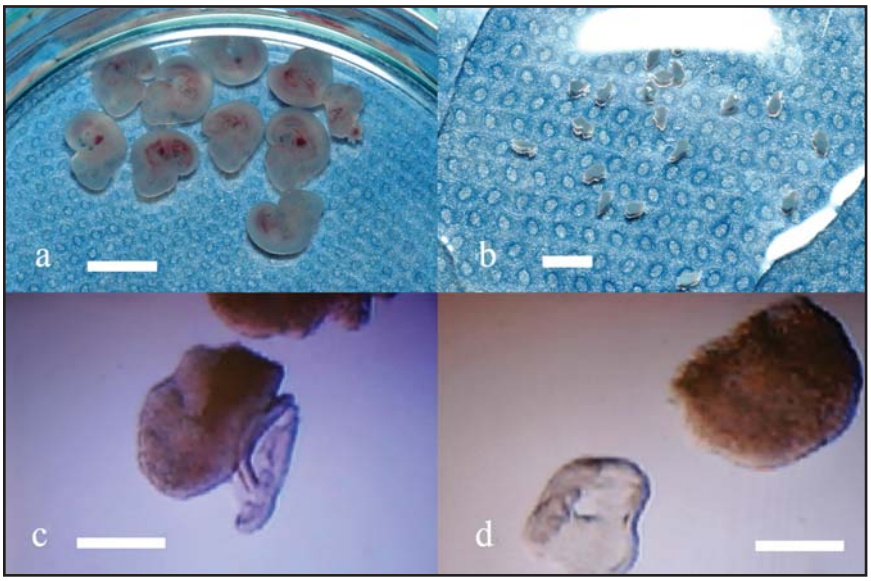

Fig. 1: Embryo dissection and ectoderm separation. a) Embryo harvest at E12.5. b) Dissected fragments are stored in PBS with Ca and Mg. c) Enzymatic separation of tissues from fragments in a pancreatin/trypsin solution, the ectoderm separates cleanly from the mesenchymal portion of the fragment. d) Competent ectoderm fragment used in experiments. Scale bars: a) 5mm, b) 300µm, c and d) 125µm.

removed from the mold and cultured *in vitro* until implantation. Most of the constructs were implanted, but some were used for preimplantation histological analysis and viability tests. Cell viability within the construct was initially assessed by means of histological analysis and confirmed by Promega live and dead cell staining (Invitrogen Live/Dead, L3224), the results of which were viewed under fluorescence microscope (Fig. 2).

Experimental constructs

Constructs were prepared in two phases, ectodermal and mesenchymal, which were separately polymerized within the mold. The ectodermal phase was constructed by loading 5 µl of the hydrogel solution containing EOE fragments (5 to 15) in the apex of the pyramidal mold. The mesenchymal phase was prepared by diluting 400,000 cells in 25µl hydrogel, pouring it over the ectodermal phase and polymerizing.

Positive Controls

Three types of positive controls were used. The tooth germ was dissected at the same gestational age as the experimental constructs and ectoderm and mesenchyme were separated. The mesenchyme was disaggregated with a dipase solution (Gibco 17105041) in PBS 2U/ml, incubated for 20 minutes and then used to produce the constructs by means of the process described above for preparing experimental constructs. A second type of positive control was prepared by dissecting the tooth germs at a more advanced gestational stage (E19.5), and including the dissected germ without disaggregating in PEGDA hydrogel. The third type of positive control was constructed by dissecting neonate tooth germs (4PN), separating the ectoderm from the mesenchyme by mechanical dissection, and re-aggregating it in a hydrogel mold.

Negative Controls

Negative controls were prepared by including 400,000 BMSC in PEGDA.

Implantation surgery

Implantation surgery was performed under sterile conditions in the operating room. The animal was placed in an induction chamber and anesthetized (Ohmeda vaporizer model Fluotec 4 using 3% Halothane with an oxygen flow between 1.5 and 2 l/min. After induction, the abdomen was shaved and disinfected with 0.2% clorhexidine solution. The animal was reintroduced in the induction chamber and afterwards connected by means of a rodent mask to an open anesthesia circuit and maintained with 1.5-2% Isoflurane (FORENE®) (Ohmeda vaporizer model Isotec 3) and a 1.5-2 l/min oxygen flow. Heart rate and oxygen saturation were monitored (Ohmeda Biox 3700 pulsoximeter®) with the pulsoximeter's probe (Ohmeda Veterinary Pulse Oximeter Lingual Sensor®) attached to the animal's tail. Heart rate was maintained at about 250 bpm and saturation over 90% during the 25-minute surgery. At normal operating room ambient temperature (21C), small rodents

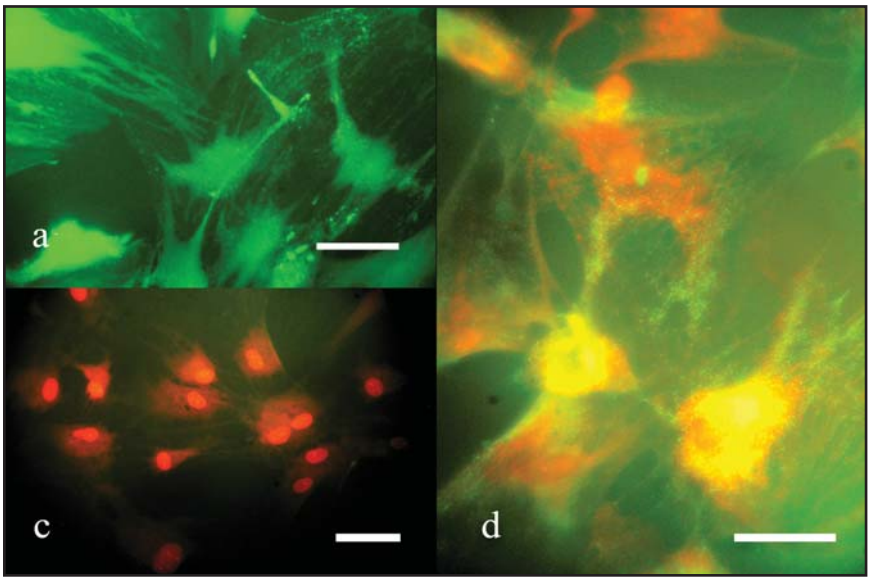

Fig. 2: Determination of pre-implantation construct cell viability using Promega's live and dead cell stain. a) Control with live cells. b) Control with dead cells. c) Mesenchymal cells seeded in a three-dimensional hydrogel (PEGDA) scaffold. Scale bars: a and c) 30µm, b) 60 µm.

can rapidly lose body temperature due to heat dissipation, therefore a thermic blanket was placed on top of the surgical table to avoid hypothermia during the surgical procedure.

To gain access to the implantation site the animal was placed in a decubitus supine position and a 1.5 cm midventral incision was performed over the Linea Alba 3 cm caudal of the xiphoid appendix. A layer dissection was performed until exposure of the parietal peritoneum, which was then cut in order to gain access to the peritoneal cavity. The peritoneum was cut and tissue forceps used to extract the ileal portion of the small intestine, which was extended on the shaved abdominal region. For implantation, two triangular areas of mesentery delineated by nutrient vessels and the intestine wall were selected (Fig. 3a), upon which a casing was made by passing a continuous 6-0 suture through the perivascular fat flanking the selected area (Fig. 3b), taking care not to strangle the vessels or break the mesentery or the construct upon closing (Figs. 3c and d). The ileum was replaced inside the abdominal cavity, the muscular and fascial layers closed in one plain with a simple continuous suture, and the skin with simple interrupted sutures both using 4-0 silk. Postsurgical analgesia was achieved by oral administration of a 0.62% morphine/water solution (2.5 mg/kg) dosed every 6 hours for 24 hours. Once the constructs had reached the selected maturation time (4-5 weeks), the animals were euthanized and the constructs recovered and fixed in a buffered formalin solution (Sigma HT501528), after which they were processed and stained with hematoxylin eosin (HE).

RESULTS

In this study, couplings to obtain embryos needed for the experiments were scheduled by estrus determination by means of vaginal wall impedance readings, which showed that 16.75% of the females sampled were in estrus, 45% were served and 27.7% carried embryos upon sacrifice. A total 34 pregnant females were euthanized humanely, from which an average 7.2 embryos/female were obtained. Embryo development was not necessarily homogeneous, and sometimes differed by up to 0.5 days. Only EOE from embryos with uniform development was used for preparing the constructs.

The whole-mount *in situ* hybridization technique showed that the expression of selected tooth initiation markers (*Pitx2 Shh and Wnt10a*) in EOE is diffuse or has concentration gradients at stages E11.5 and E12, and is more localized to the areas of future dental development during gestational stages 12.5 and E13.5. Prior to this stage, the signal is distributed diffusely in the first and second branchial arches ectodermal tissues. Similarly, during the development stages observed, the *Shh* and *Pitx2* signals, particularly the former, are located in the limb formation control centers (ZPA) appearing first in the front limbs (E11.5) and later in the hind limbs (E13.5). The concentrated expression of these three genes was indicative of the appearance of tooth initiation signals, defining the moment for harvesting embryos to be used in construct preparation. The dissection of the mandibular segment of the first branchial arch and its subsequent enzymatic treatment allowed ectodermal and mesenchymal portions to be separated, and the EOE was used in the

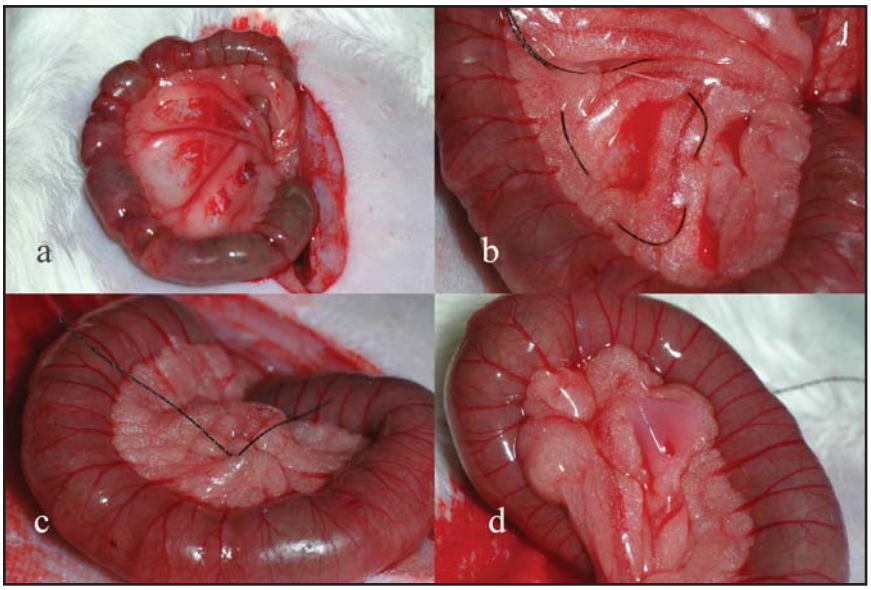

Fig. 3: Construct implantation. a) The terminal mesenteric vessels (arteries and veins) fan out and with the wall of the small intestine delimit triangles of mesenterium. b) A continuous 6-0 suture was passed through the perivascular fat adjacent flanking the triangle and forming the casing. Extreme care was taken so that upon closing the suture would not strangle the nutrient vessels. c) Closed casing containing the construct. d) Construct in position surrounded by mesentery and nutrient vessels.

experiments to induce tooth differentiation. The inclusion of these fragments as a group in a small volume of hydrogel formed the ectodermal phase of the constructs, which was subsequently attached to the mesenchymal phase, constituted by bone marrow purified cells cultures used as initiation signal receptors.

Purification of the sub-population of stem cells was based on their ability to adhere to the surfaces on which they were seeded, expanding in two directions, and hematopoietic cells were progressively excluded by means of successive medium changes. A homogeneous cell population was obtained at second passage, and at this stage they were used to produce the three-dimensional constructs. Including 400,000 BMSC's in a minimum hydrogel volume of 25 µl allowed the production of a three-dimensional structure with no polymerization defects and easy to manipulate during the *in vitro* culture and *in vivo* implantation processes. Figure 2 shows live cells remaining in PEGDA constructs after three days' culture *in vitro*. These results guided the production and implantation of 76 constructs; 37 experimental (17 at E12.5 and 20 at E13), 12 positive controls and 27 negative controls, in the ileal mesentery where they matured for 3 to 5 weeks surrounded by vessels and perivascular fat. The selected implantation site facilitated the recovery of the constructs and dissection of the surrounding tissues.

Histological analysis of the experimental controls showed that the EOE remained viable and proliferated, becoming invaginated within the hydrogel, while the mesenchymal cells did not. In order to enable closer contact among BMSC population in an attempt to stimulate them to maintain viability, a hollow pyramidal PEGDA mold was designed (Fig. 4a) that allowed for condensation of the BMSCs over the ectoderm fragments by means of a brief centrifugation prior to hydrogel polymerization. The constructs thus formed were maintained in culture for 7 days (Fig. 4 b), and then observed using an inverted microscope. Contraction of the cell mass towards the base of the pyramid was detected (Fig. 4c). Although concentration of BMSCs was increased, thus increasing cell density in the hydrogel/cell solution before polymerization, results did not improve cell viability in the implanted constructs (Fig. 4d and e). Non-proliferation of BMSCs in PEGDA was confirmed with results of the negative controls, all of which showed the BMSCs undergoing cell death (Fig. 4f).

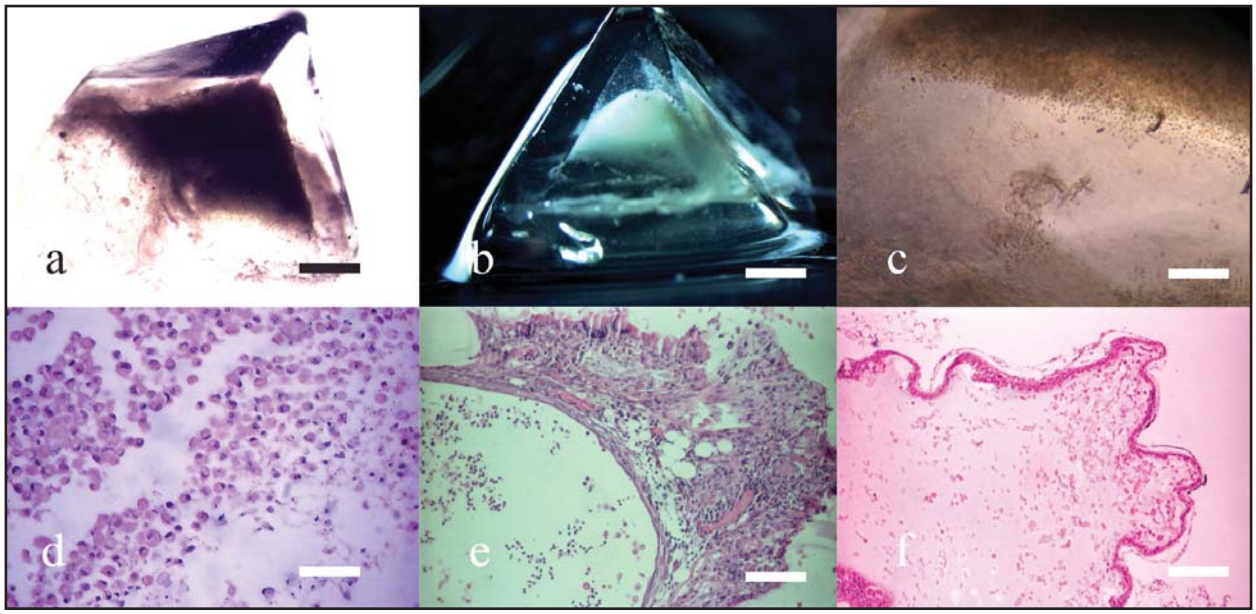

Fig. 4: PEGDA constructs. a) Hollow pyramidal mold. This hollow mold allowed BMSCs to be condensed over ectodermal cells with gentle centrifugation before polymerizing the hydrogel. b) Pyramid with hydrogel loaded with BMSC and polymerized inside the hollow mold, after being cultured in α-MEM for 7 days, there is a contraction in cell mass towards the base of the pyramid. c) Inverted microscope image of the BMSC culture embedded in hydrogel; picture taken of the pyramid in Figure b. d) Histological section of the BMSC from the previous picture stained with hematoxylin eosin (HE), cells show all phases of anoikis (karyopyknosis, karyorrhexis and karyolysis) e) Section of an experimental construct recovered at 5 weeks, showing the vital proliferative ectoderm. The mesenchymal portion of the construct is occupied by cell remains. f) Section of a negative control recovered at 5 weeks, picture of the mesenchymal portion similar to the one in e, HE stain. Scale bars a and b) 1 mm, c and e) 50µm, d) 25 µm and f)150 µm.

In some complete experiments, where the epithelial portion was in direct contact with the mesentery, an external vascular network was created (Fig. 5a) extending into the ectodermal portion, and some cells of the mesenterium acquired a columnar morphology similar to that of odontoblasts at the beginning of dentin formation (Fig. 5b).

In the positive controls, histological sections show that the hydrogel allows nutrient diffusion but prevents the vascular proliferation that greatly contributes to final tooth development. Vascular proliferation only occurred in cases in which there was a gap in the hydrogel cover as a result of construct breakage at the time of implantation.

In the (E19.5) positive controls, tooth germs were included within the PEGDA (Fig 6a) but there was involution of the odontogenic complex after implantation, leading to the formation of a epidermal inclusion cyst-like structure (Fig 6b) with an ectodermal portion of keratinized stratified squamous epithelium and a defined lumen containing shedded keratin (Fig. 6c) and (Fig. 6d). At E12.5, when the posterior segment of the mandibular portion of the first branchial arch was embedded in hydrogel and implanted, a circular primordium formed with stellate reticulum-like cell structure surrounded by ectodermal columnar cells, considered to be nodular structures predecessors of dental structures (Fig. 6e). When a four-day post-natal (PN4) germ was used as positive control, interaction was achieved between BMSCs and EOE, forming a structure with dental tissues but lacking clearly defined organization (Fig. 6f).

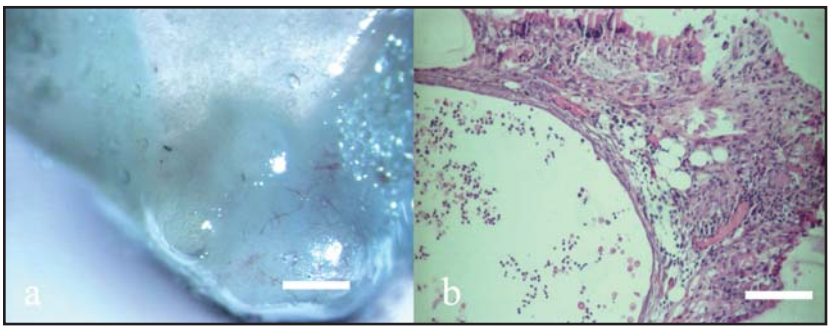

Fig. 5: Experimental construct at 5 weeks' implantation. a) View of an external vascular network associated with the epithelial portion in direct contact with the mesenteric membrane. b) The vascular contribution is distributed within the ectoderm. The mesentery cells in contact with the embryonic ectoderm acquire a columnar morphology, HE stain.

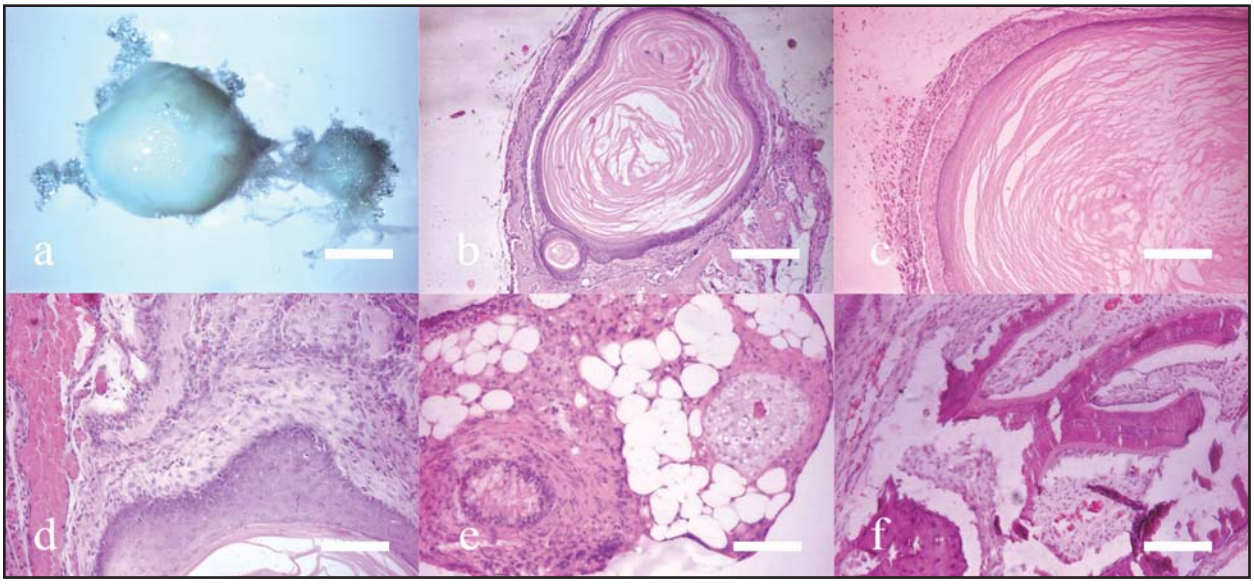

Fig. 6: Positive controls. a) Macroscopic image of a positive control of an E19.5 tooth germ embedded in hydrogel and implanted in the mesentery for 5 weeks. b, c and d) Histological images of the specimen showing a layer of keratinized stratified squamous epithelium. The lumen is filled with sloughed keratin, HE stain. e) Nodular structures which are precursors of tooth structures in a positive control made with an E12.5 germ, implanted for 5 weeks. A circular primordium can be seen, which contains a stellate reticulum-like cell structure surrounded by columnar ectodermal cells. f) Positive control with a 4-day post-natal (PN4) germ. Deposition of irregularly shaped matrix of enamel and pre-dentin. Scale bars: a) 500µm, b) 300µm, c) 100µm, d and e) 30µm, f) 60µm.

DISCUSSION

Tooth production by means of tissue engineering is an important aim in dental research, which is why this study seeks to establish three-dimensional micro-environments establishing communication pathways among different cell types. Those interactions would be homologous to the ones occurring naturally during tooth development, enabling the regeneration of dental tissues. A tooth is the result of the differentiation of epithelial cells into ameloblasts and of ectomesenchymal cells into odontoblasts. These differentiated cells produce enamel and dentin matrix[13] through sequential, reiterative, and reciprocal interactions of signaling molecules between the ectodermal and mesenchymal tissues[1]. Induction of the signaling cascade using competent embryonic ectoderm and mesenchymal tissue of non-dental origin has been extensively described[14], demonstrating that in early stages of mouse development, the epithelium covering the stomodeum of the mandibular portion of the first branchial arch provides inductive signals for tooth morphogenesis and histodifferentiation to non-dental origin mesenchyme suggesting that ectomesenchymal cells are plastic in their responses to signals form the oral epithelium. There are two main approaches to tooth regeneration; the first based on tissue engineering, which seeks to regenerate teeth by seeding disaggregated tooth germ cells on biomaterials that serve as scaffolds, while the second involves reproducing the development processes in tooth formation using embryonic dental tissues as inductors[15]. This study combined the two approaches by seeding embryonic cells and stem cells from adult individuals in a PEGDA scaffold which was subsequently implanted *in vivo* in rats. PEGDA hydrogel was selected as the scaffold because prior research has enabled the *in vivo* production of a structure in the shape of a human mandibular condyle from a population of mesenchymal stem cells induced to differentiate into chondrogenic and osteogenic lineages[12] allowing stratification of embryonic ectodermal tissues and mesenchymal BMSC by polymerizing separate layers which maintain the assigned spatial relation. This property was the main reason why PEGDA hydrogel was selected as the scaffold for producing the constructs.

In order to obtain the 234 embryos used, scheduled matings were performed using females that tested positive for estrus by means of vaginal wall impedance reading, improving the service/pregnancy ratio and contributing to the application of the 3Rs principle in animal model research, since by reducing pseudo-pregnancies the number of available females for periodical sampling remained stable[16].

Different studies have used mouse competent embryonic dental ectoderm in tissue engineering experiments, to induce differentiation in adult bone marrow-derived stem cells, achieving *in vivo* formation of complex dental structures[17, 18]. Collecting ectodermal cells from rat embryos offered advantages because due to their size, the cell volume harvested is greater, tissue manipulation during the micro-dissection procedures is easier and it facilitates future implantation of the germs obtained by cell recombination in individuals of the same species. However, there is little information in the literature regarding embryonic tooth development and the appearance of putative initiation signals for the formation of teeth in rats, which is why it was necessary to use the whole-mount *in situ* hybridization technique to evaluate the location of the expression of three putative signaling molecules in embryonic oral ectoderm associated with dental competence. In *Lewis* rat embryos at different stages of development, the results showed that there is a delay of approximately two days in the appearance of initiation signals compared to the information reported for mice. While in mice, the initiation signals, especially the expression of *Wnt10a* and *Pitx2* are concentrated in the embryonic ectoderm that covers the stomodeum at E10.5, in rats this concentration is expressed at E12.5. This is why the ectoderm covering the stomodeum of the mandibular processes of the first branchial arch in Lewis rat embryos, between E12.5 and E13.5, is competent for inducing in the underlying mesenchyme the signaling cascade needed for the formation of dental structures. Careful scheduling was needed to coordinate the time at which ectodermal tissue was harvested and the progress of the mesenchymal cultures because as from the date of the scheduled crossing, there were 12.5 to 13.5 days to have the BMSCs in second passage.

The implant sites reported in other studies were selected based on criteria of immunoprivilege, surgical approach, presence of nutrient contribution and ease of implant recovery. The most frequently cited sites include kidney subcapsular space, grater omentum, intraoclular space and subdermal pockets[19,20]. In this study mesentery was selected as the construct

receptor site because the surgical approach is simpler, it has plentiful nutrient contribution and construct recovery is easier, and the use of subcapsular space avoided because it limits the size of the implants that can be placed and also because in the present study immunoprivilege did not play an important role, considering that an isogenic strain was used both as source of tissues and construct receptor. Previous studies have reported the use of extracellular matrix (ECM) from decellularized mesenteric membrane as scaffold for cell inclusion in tissue engineering. The ECM is a complex network of collagen, fibronectin and other proteins intermeshed with proteoglycans[21] which serves not only as support material but also regulates cell functions such cell proliferation, migration and differentiation[22] and modulates the transduction of signals activated by various bioactive molecules such as growth factors and cytokines[23]. Reports published after the initiation of this study, mention the mesenterium as an appropriate receptor site for implanting islets of Langerhans in experiments pursuing recovery of pancreatic function in animals affected by diabetes mellitus[24]. Other study reported implantation of hepatocyte progenitor cells in the mesenteric leaves of animals, enabling them to proliferate and fill the spaces of the macroporous structure, suggesting the feasibility of establishing three-dimensional cultures of hepatocyte progenitor cells for hepatic therapies based on tissue engineering[25].

The histological evaluation of the implantation of experimental, positive controls and negative control implants shows that the selected site facilitates cell interactions by providing nutrients that ensure cell proliferation, and sometimes, when the ectodermal cells in the construct made contact with the mesentery, complex multicellular structures were formed with a well-developed vascular network. The EOE included in the constructs remained viable throughout the implantation period but in spite of the fact that the hydrogel allows the proliferation of differentiated cells, its physicochemical characteristics promote stem cell death[26]. In some specimens and as a result of cell degradation, this produced cystic spaces surrounded by chronic inflammatory infiltrate. The histological analysis of the negative controls showed that in all implanted constructs, the BMSCs underwent a process of cell death characterized by karyopyknosis, karyorrhexis and karyolysis, which was reported after the completion of the experimental phase of this project[26] and has been called anoikis, (apoptosis induced by lack of interaction with proteins of the extracellular matrix). In all the negative controls that were recovered, the BMSC site showed no vascularization, suggesting that the hydrogel may act as a barrier that allows nutrient diffusion but at the same time prevents vascularization, for which direct contact between the mesenteric membrane and implanted cells is needed.

A different situation was observed in the positive controls because the germ included in the hydrogel was made up of mesenchymal tissue with mature extracellular matrix supporting the cells. At (E19.5) there was involution of the odontogenic complex leading to the formation of a epidermal inclusion cyst-like structure with a defined lumen containing sloughed keratin and an ectodermal lining of keratinized stratified squamous epithelium, indicating that further development of the cultured germ does not ensure completion of morphogenesis and histodifferentiation processes. The timing of germ harvesting and the presence of small quantities of non-dental tissue may contribute to this situation, as previously reported[27]. At PN4, cell interaction was achieved and a structure containing dental tissues lacking clear organization was formed. Finally, histological assessment of the experimental constructs showed that EOE remained viable and proliferated, invaginating in the hydrogel. However, as in the negative controls, the mesenchymal cells underwent anoikis, which was the determining factor in the absence of dental tissue formation when the two cell types were included in a PEGDA hydrogel matrix. A frequent histological finding in the experimental constructs shows that mesenchymal mesenteric cells in contact with the embryonic ectoderm acquired a columnar morphology similar to odontoblasts at the beginning of dentin formation. This may be related to the ability of the mesentery ECM to foster mesenchymal cell proliferation and differentiation when it comes into contact with EOE, by contributing growth factors, cytokines and vascularization to the tissue.

Nodular structures of ectodermal origin described as dental structure precursors[28] were often found in the E12.5 positive controls. This demonstrates that under certain circumstances there may be cell interactions inside the scaffold, but they stop before dental tissues differentiation at the selected implantation times. Explanations on why this happens can relate

to the fact that initiation signals at the selected harvesting stage are not strong enough or the EOE cell volume included in the scaffold is deficient and both imply insufficient intensity of initiation signals necessary to induce differentiation in the BMSCs[27]. Finally, during BMSC culture, it might be necessary to supplement the medium with growth factors and cytokines related to mesenchymal competence (signaling molecules that control gene expression in the mesenchyme) and which would complete or reinforce the signals required for differentiation towards the dental lineage. The use of other types of supporting matrices such as collagen gel or sponges to support tooth germ cells dissociated in later development stages has been reported, and they have been shown to foster cell interactions. Their use in the case of early interactions such as those that occur between the BMSC and EOE should be examined in order to achieve an ideal dental primordium.

CONCLUSION

PEGDA hydrogel supports the viability and proliferation of EOE but not of BMSC. The latter undergo apoptosis, preventing the existence of a cell group that could act as a receptor for the initiation signals originated in the ectoderm necessary for tooth formation. The search must continue for an ideal support that will maintain the three-dimensional stratification of cells, enable their manipulation during implantation, and facilitate vascular infiltration.

ACKNOWLEDGMENTS

This study was funded by Colciencias and the Academic Vice Rector's Office of the Pontificia Universidad Javeriana. The authors would like to thank Dr. María Beatriz Ferro, Academic Dean of the School of Dentistry of the Pontificia Universidad Javeriana for her invaluable support in conducting this study, Dr. Irma Thesleff for her generous donation of the plamids and for training the researchers in techniques necessary for the development of this project and, DICHCOL SAS. for their contribution to the special sterilization protocols used for this study and Mrs. Olga Lucía Beltrán for her conscientious care of the colony.

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