Evaluation of Ethylene Oxide, Gamma Radiation, Dry Heat and Autoclave Sterilization Processes on Extracellular Matrix of Biomaterial Dental Scaffolds

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Research Article

Keywords: sterilization, decellularization, scaffold, tissue engineering, and extracellular matrix

Posted Date: September 1st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-834563/v1

EVALUATION OF ETHYLENE OXIDE, GAMMA RADIATION, DRY HEAT AND AUTOCLAVE STERILIZATION PROCESSES ON EXTRACELLULAR MATRIX OF BIOMATERIAL DENTAL SCAFFOLDS

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ABSTRACT:
Scaffolds to receive stem cells are a promising perspective of tissue regeneration research, and one of the most effective solutions to rebuild organs. In the near future will be possible to reconstruct a natural tooth using stems cells, but to avoid an immune-defensive response, sterilize the scaffold is not only desired but also essential to be successful. A previous study from the group, confirmed the insertion of stem cells extracted from rat's natural teeth, and implanted into the alveolar bone, could differentiate themselves in dental cells, but the scaffold's chemistry, geometry, density, morphology, adherence, biocompatibility and mechanical properties remained an issue. This study intended to produce a completely sterilized dental scaffold with preserved extracellular matrix. Sixty samples were collected, kept in formaldehyde, submitted to demineralization and decellularization processes and sterilized using four different methods: dry heating; autoclave; ethylene-oxide and gamma-radiation. They were characterized through colorimeter scale, optical images, radiography, micro-hardness, XRD, EDS, XRF, SEM and sterility test. The results evidenced the decellularization alone is not enough to eliminate microorganisms from dental scaffolds, and the four sterilization methods were fully effective with preservation of ECM. The dry heat and autoclave could be detached from others because of cost-benefit, but ethylene oxide or gamma radiation should not be discarded mainly if it is considered other possible applications.
KEYWORDS: sterilization, decellularization, scaffold, tissue engineering, and extracellular matrix.

1. INTRODUCTION:

Tissue engineering is one of the most challenging areas of regenerative medicine, more than replacing damaged tissues, expecting to contribute in restoration, maintaining or improvement of the tissue[1]. To determine the suitability of scaffolds, it is important to consider: biocompatibility, biodegradability, mechanical properties, scaffold architecture, and manufacturing technology. The recovery of organic functions, through the use of stem cells, constitute the most challenging tasks of regenerative medicine, the ideal substitute is from autologous origin[2].

Biological and synthetic materials are also used as scaffolds, such as collagen, chitosan, alginate, and proteoglycans. The natural polymers are more biological active, and usually promote excellent cell adhesion and growth. The biodegradability also presents the advantage of allowing host cells, consequently, the production of its' own extracellular matrix.

Polymers can be tailored built and also control the biodegradation through the composition, but the risk of rejection due to reduced bioactivity remains an issue[3–5].

Hydroxyapatite has better mechanical properties but less suitability for cell infiltration and vascularization. Although ceramic scaffolds exhibit excellent biocompatibility, due to similarity with hydroxyapatite (HA), the applications are limited by the brittleness and difficulty sharp, in addition new bone are formed in a porous HA cannot sustain the mechanical loading needed for remodeling[6].

The structure, functionality and mechanical properties, mainly when producing scaffolds from single phase biomaterial, encouraged research with composite materials, as ceramic-polymer based scaffolds[7–9].

The native Extracellular Matrix (ECM) can influence a cell behavior, and the mechanical properties are critical to mechanic transduction, development, differentiation and regeneration[10,11].

The scaffolds are tridimensional structures similar to original tissue, in texture, macro and micro-geometry making easier the cellular adhesion and consequent expression[12].

Collagen sponges and gels used for tooth regeneration has shown great compatibility to retain cells and support proliferation and differentiation, also can stimulate the formation of calcified tissues[13].

Dental pulp stem cells, seeded on collagen scaffolds (in vitro), can produce a fresh stemmed pulp tissue[14,15].

The complete removal of cells from a tissue or an organ (decellularization) is a complex process, as there are structural and functional proteins constituting the ECM, if it is damaged, can compromise the adequate expression of stem cells. The demineralization and decellularization solutions may interfere on ECM and alter its ultra-structure, on the other hand, if biological contents are not completely removed, can cause an inflammatory or immune response against this foreign body. To avoid damages to the dental structure, preserve the ECM and completely remove thecells, were chosen commonly used cleaning and sanitizing substances.

To reach the teeth's pulp chamber and connective tissues (vessels, arteries and nerves)

is necessary a demineralization process, removing the calcium from the mineral tissue, allowing the action of cleaning substances. One alternative way to remove the pulp tissue could be an endodontic access, but this kind of intervention would damage the dental scaffold permanently, reason why was avoided.

Scaffolds can be based on biomaterials to be used in tissue regeneration with ability to carry out cellular activities, drugs and genes, produced with biocompatible material, absorbable or not[16]. Used to regenerate tissues or organize cells, they help on the production of their new extracellular matrix. As the structure is very complex, being composed by solid, fiber and gel, the best scaffold would be the natural one because it keeps living cells in activity.

The purpose of mimicking this scaffold is to study the porosity and consistency, what means to copy its macro and micro-geometry, Gupte & Ma[17] defends the materials often bio-mimic scaffolds' features, trying to imitate ECM, developing characteristics for cell-bearing and create an environment to improve cell adhesion, proliferation, differentiation and also to promote the tissue regeneration.

More than design, the mechanical integrity and functionality, morphology and surface compatibility are essential for cell adhesion, differentiation, and integration with surrounding natural tissues. Additional care need to be taken with sterilization, to avoid infection and rejection[18–20].

Although good results are reached developing mineralized dentin, enamel and crown through the use of tissue engineering, the complex structure and interactions between the epithelium and mesenchyme tissues justify the difficulty to develop a viable organ from stem cells[1]. Lack of vascularization in scaffolds and tissue engineered constructs is a major problem[21,22], an alternative to vascularization is engineering microvasculature by stem cells previously to implantation[23,24].

Decellularized tissues are extensively used to obtain biomaterial scaffolds, the efficiency of cells removal depends of the origin and the methods used to sterilize it. The ideal conditions to use these tissues include the minimum interference on biochemical composition, ultra-structure and the mechanical behavior of ECM[25,26].

The elimination of microorganisms as bacteria, spores, protozoa, fungi, and virus are essential to reach an acceptable level of safety on sterilization[16]. Autoclave sterilization (AS), ionizing irradiation, dry heat (DH), ethylene oxide (ETO), peracetic acid, peracetic-plasma acid, formaldehyde and formaldehyde vapor are very explored sterilization methods[27]. The sterilization is a key step on biomaterials procedures, infections must be avoided to not cause troubles, or eventually the death of patient.

The organ to be sterilized drives the sterilization method choice, the autoclave is the most used in dental clinics, the thermo-sensitivity of several hospital materials do not allow the generic use of this method[27]. A second method is the dry heat, which uses high temperatures, what can promote the hydrolysis and/or the matrix fusion, compromising the biocompatibility. An alternative method could be the chemical gas sterilization through ethylene oxide, this method is very effective at low temperatures, however, the gas residues are alkylation agents, what can modify

and/or compromise the properties of the biomaterial[28].

Host's reaction to a foreign body can be inflammatory response or an immediate and immune rejection is a reason to search a biomaterial scaffolds from decellularization of tissues and organs. The conservation of ECM are well tolerated by xenogeny receptors, to use biomaterial scaffolds, it is needed an adequate sterilization process to be implanted[29].

The restoration of tooth loss is a great challenge in dentistry, and the search for a biological substitute envisages new therapeutic horizons. There are several mechanisms for demineralization, decellularization and sterilization of the organs, but was not found a definitive process for the dental scaffold. Dentistry aims not only on restoration but also on its regeneration, justifying the need of advancing in studies to mimic the teeth enabling it as biomaterial scaffold, what also will demand an efficient sterilization process.

A previous study from the group presented, after demineralization-decellularization, remaining pulp on almost 30% of the samples. It could represent a considerable risk to rejection or inflammatory response, reason why the sterilization methods were introduced in this phase of the study[36]. The main objective was to verify which of the presented methods would be the most effective on sterilizing extracted natural teeth, enabling them as biomaterial scaffolds to receive stem cells.

2. MATERIALS & METHODS

The study protocol was in accordance with relevant guidelines and regulations including the Declaration of Helsinki, and approved by the Ethics Committee in Research of Federal University of São Paulo (UNIFESP), approval number 1529/2015. Written informed consent was obtained from all patients, including statement from legally authorized representatives of minor participants.

Sample Preparation and sterilization treatment:

Sixty pre-molar teeth from healthy patients (age from 14 to 45 years old) composed the studied samples, were excluded all sectioned, bleached, with any kind of coronary wear, anomalies on mineralized structure, teeth from patients who declared infect-contagious disease or chronic use of drugs. Only teeth with complete root formation were included[12].

After extraction the teeth samples were kept at room temperature in a 10% formalin solution, were washed in de-ionized water, dried at room temperature (24 hours), packaged in surgical grade paper and randomly separated in groups: control group GF0 (1-10) – was not submitted to any kind of sterilization (kept in formaldehyde);

The remaining fifty samples were submitted to demineralization and decellularization processes.

Scaffold demineralization-decellularization

Fifth experimental samples were demineralized and decellularized with Phosphate Saline

Buffer (PBS) and 28g of EDTA, 9% hydrogen peroxide and enzymatic detergent, they were washed in running water, kept in sterile water, dried at room temperature for 24 hours and packaged in surgical grade paper to be sterilized. In accord to protocol developed in the first phase of this study[36].

During the demineralization process, the samples were weighted weekly at Shimadzu high precision equipment model AY220. As the size and weight of the teeth varied significantly, the percentage variation was carried out, comparing the losses divided by initial weigh.

Because of huge difference in weight between formaldehyde group and decellularized group, and to allow the directly comparison of different sterilization processes, the control group demineralized and decellularized samples was prepared, nominated as GD4 (41-50).

Dry Heat - GD1 (11-20)

The samples were treated in an oven - at 170 °C by 120 minutes;

Autoclave – GD2 (21-30)

Temperature range from 60 to 129 °C, and pressure between 1.7 to 1.8 kg/cm^2. In two steps, the first 20 minutes sterilizing, and 30 minutes drying.

Ethylene Oxide – GD3 (31-40)

The packed samples were sterilized through exposition to an ethylene-oxide (30%) + CO_2 (70%) atmosphere at a relative humidity from 40-70%, pressure range of 0.4 to 0.5 kgf/cm^2, exposure time of 4h with temperature range from 45 to 55 °C.

Gamma Radiation – GD5 (51-60)

Packed and sealed samples were exposed to radiation of 25 kGy with multipurpose irradiator of ^{60}CO (CTR / IPEN / CNEN-SP)

Characterization:

The samples were characterized to analyze the molecular structure of ECM, confirm the preservation of shape (drawing), atomic composition, mechanical resistance and the efficacy of sterilization processes. During demineralization-decellularization processes, to reach maximum cleaning without removal of large plates, a weekly weight measure was proceeded at Shimadzu equipment model AY220. Because of the difference in size and weight of natural teeth collected, the losses comparison was done using percentage variation $\Delta m\% = (1 - (mf/mi))*100$, where $\Delta m\%$ = variation in percentage of mass, mf = final mass, and mi = initial mass.

ECM preservation:

External drawing and color - The teeth were photographed (digital camera cannon model EOS Rebel T100) and radiographed in the longitudinal plane, before and after the

demineralization-decellularization and sterilization processes, using a positioning device to keep uniform distance. On radiographs was used an aluminum scale in order to standardize the different shades of gray observed, where the colorimeter Chroma Meter CR-400 - Konica Minolta Camera Co., provided a quantitative measurement, evaluating the color variation after sterilization processes.

ECM structure – To verify the micro-structural interfaces (enamel, dentin, cement and pulp chamber), the samples were carefully sliced, and analyzed at SEM equipment (EVO MA 15 Zeiss equipment), were the samples were fixed on stubs and covered with exploded carbon tape, a thin carbon tape established the electrical contact between the sample surface and the sample-holder.

To confirm the atomic composition, an EDS analysis was proceeded at the same equipment (EVO MA 15 Zeiss equipment), and also was used an XRF (X-Ray Fluorescence) measurement, in a Bruker S2 Ranger equipment, adjusted to analyze a 10 mm diameter and calibrated using pure Cu standard.

In order to analyze the crystallographic arrangement of atoms, the samples were ground into powder using pistil and mortar and measured in the XRD equipment D8 diffractometer, with linear position detector type LynxEye from Bruker. The powder was carefully positioned at sample-holder (PPMMA – Poly-methyl-methacrylate) and measured at room temperature under Cu kα radiation, wavelength 1 = 1,5406 Å, 2θ scanning from 20° to 120°, step of 0.02° and exposition time of 1.2s. The data were analyzed through Rietveld Method[30] at Topas 4.2 software from Bruker AXS, and CIF (Crystallographic Information Files) were obtained from ICSD (Inorganic Crystal Structure Database).

Mechanical Resistance – The samples were previously embedded in acrylic resin and polished, and then micro-hardness was measured at Shimadzu HMV 2T equipment, using 490.3 mN, 0.05 load, and 10 seconds.

Sterility Efficacy:
Microbiologic Test – To verify the viability of microorganisms' growth (fungi, anaerobic and aerobic bacteria), solutions of Thioglycolate (THIO) and Tryptone Soya Broth (TSB) were used. Negative control sterilization tests were proceeded using two sterilized empty tubes, which received both solutions. The positive tests were performed on 2 inoculated empty tubes, and 24 samples (control Group F0, D1, D2, D3, D4 and D5), with THIO and TSB. All tubes were incubated at 37.5 ° C for 14 days.

Histology - The dental organs were included in resin block, sliced (2 elements of each group), polished and identified. The samples were colored with Hematoxiline Eosine (HE), Masson Goldner (MG) and Steven Blue (SB), and the pictures were obtained using Inverted Optical

Microscopy Axiovert 40C (Carl Zeiss) through Axio Vision 4.1 software.

The data were treated with Friedman Variance, Kruskal-Wallis, Chi-Square and exact Fisher tests for statistical validation. The null rejection hypothesis level was set at 0.05 or 5%.

3. RESULTS

After decellularization process, almost 30% of samples still presented biological residues at the pulp chamber, repeating the results previously obtained on the demineralization study[26].

ECM preservation – The characterization techniques were used to confirm the preservation of ECM in terms of shape, atomic composition, diffractive information about atoms arrangement with the crystal and the mechanical resistance of the samples.

The mass losses (percentage variation) was lower than 1.4% between the different processes of sterilization (0% on control group, 4,2% on Dry Heat, 2,8% on Autoclave, 3,0% on Ethylene Oxide and 3,5% on Gamma Radiation), suggesting low impact on ECM, and the H value of 5.5385 ($p = 0.0186$) indicates high significance.

The optical images (photography, radiographies) observation indicated the different sterilization techniques do not affect the external shape of samples, and also no fracture were detected. Only minor changes on color was detected when compared through the colorimeter, mainly on gamma radiation group, however, apparentlynot affecting the viability of the sample as scaffold.

Analysis at SEM, Fig 1, provide details of teeth's structure with no visible variations between the different sterilization processes, were kept individual sections structures (pulp, cement, dentine and enamel) except by a visible effective action on the pulp of autoclave and ethylene oxide (EtO) methods, suggesting they have deeper actuation, although not enough for complete removal. Even some methods are able to reach the pulp chamber, none of the treatments affected the integrity of canaliculated irrigation structure.

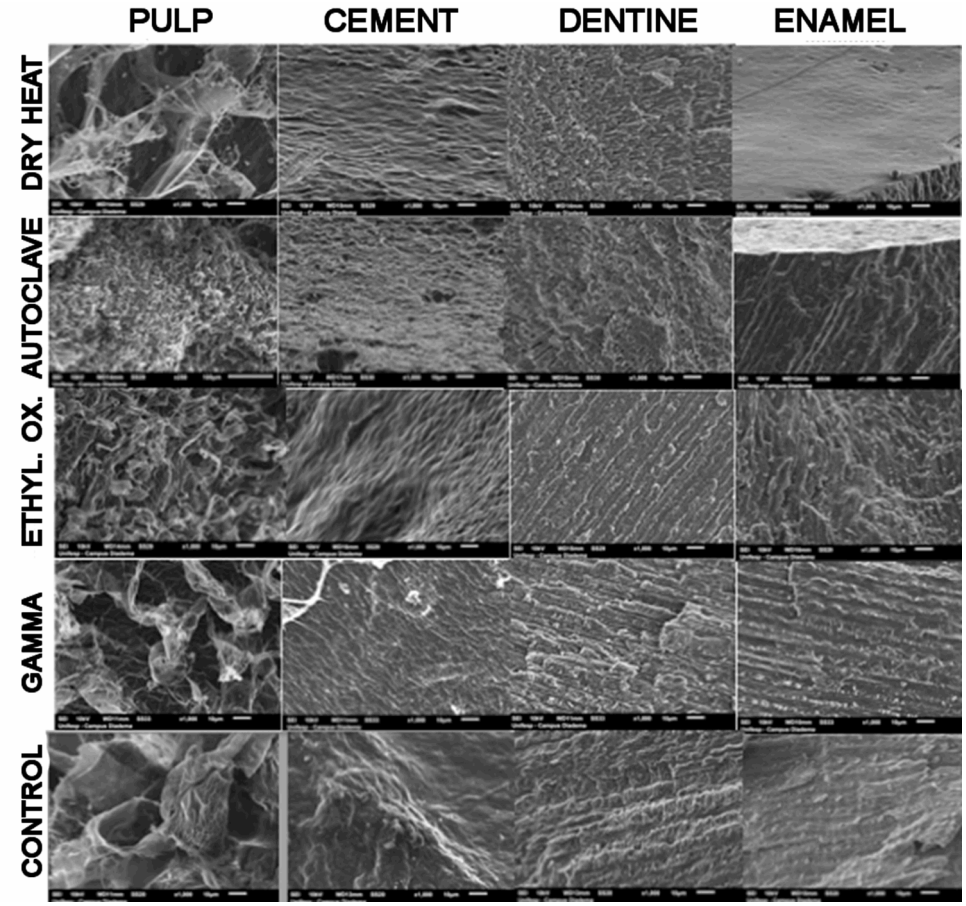

Fig 1 – SEM images do not evidence visual changes on structural regions of the teeth, preservation of canaliculated structure and effect of EtO and Autoclave on residual Pulp.

All sterilization techniques caused elevation on hardness, where Gamma Radiation (GD5) is the least impacting, while the most impacting one was dry heat (Fig 2 presents the comparative measurements). All samples presented small cracking lines, although not interfering on the final results, a further study should be interesting to confirm if it would not cause troubles in terms of resistance or cells adhesion or even reconstruction of the tissue on long term evaluations.

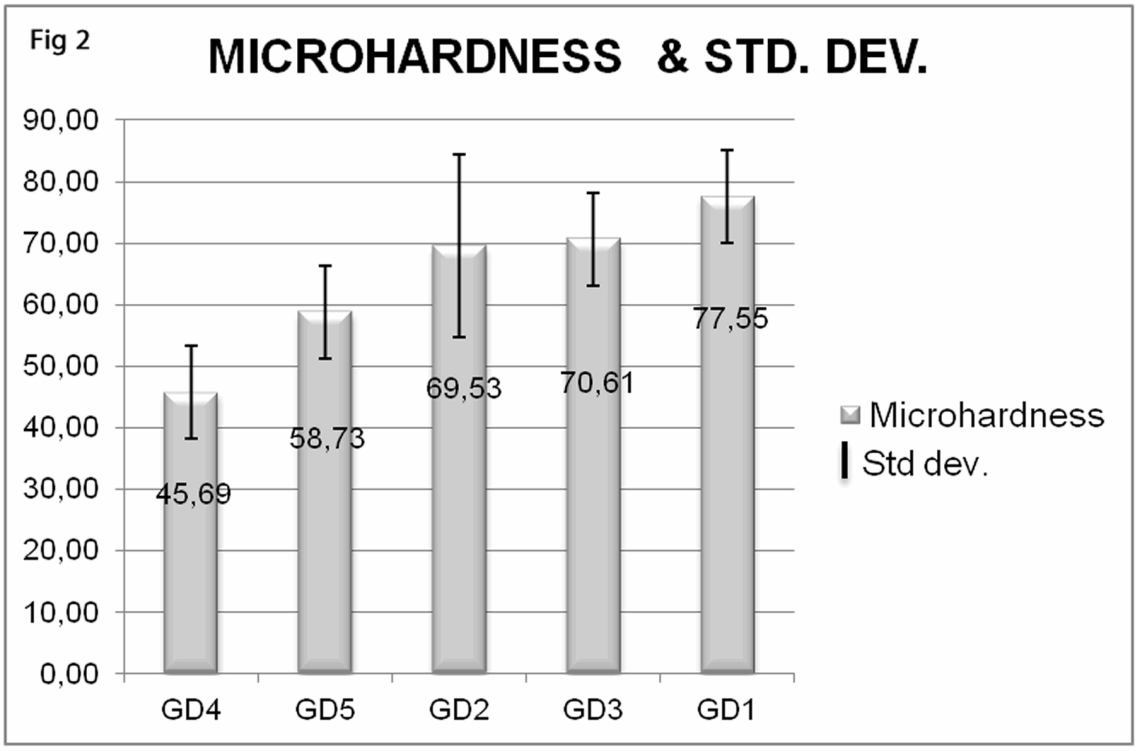

Fig 2 – Micro-hardness measurements comparing the sterilization methods and the control group, bars represents the average hardness and the lines represent the standard deviation. The H value is highly significant 52.5432 (p = 0.00001).

The Rietveld analysis of XRD data suggests the crystalline structures do not change significantly, represented by the peaks remaining on same angles even after the sterilization processes and the refining parameters GOF 1.40 and RwP = 8.51, Fig 3a presents the angles where were detected the diffraction of X-ray beans, being coincident to main compound of teeth, the Hydroxyapatite, used as reference for refinement.

The FRX analysis, Fig 3b., naturally indicates higher proportionality of Ca on GF0 when compared to demineralized and decellularized groups (GD1 to GD5), justified by significant loss of mass on demineralization process, reason why was used a secondary comparative control group (GD4).

The Ethylene Oxide group (GD3) presented the slightly higher losses of Ca, suggesting this method affects a little bit more the Ca presence than other methods, followed by Gamma Radiation (GD5), on the other ha and GD1 and GD2 methods basically do not affect the chemical composition. The hypothesis of structural arrangement modification was not confirmed by XRD analysis.

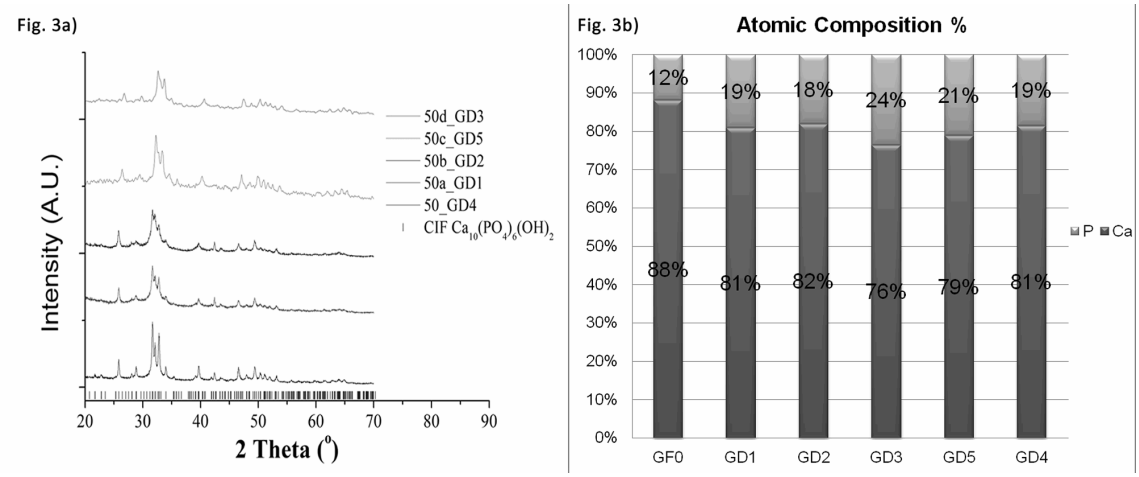

Fig 3 – a) XRD analysis did not evidence modifications on crystalline structure; b) XRF evidenced a significantly impact of demineralization process (GF0 x GD4), almost no changes on GD1 and GD2 groups, and slight higher losses of Ca on GD5 and mainly on GD3 groups.

Sterility Tests:

The negative control (NC) test with THIO and TSB solutions confirmed there were no contaminations on solutions or tubes.The positive control (PC) test with the four sterilized groups evidenced the absence of microorganisms, however the control group (GD4) and the inoculated tubes (PC and NC) presented proliferation of microorganisms, validating the hypothesis the samples and solutions were not contaminated, and the sterility tests effectiveness. (Table 1).

	TIO	TSB
NEGATIVE CRTL (NC)	-	-
POSITIVE CTRL (PC)	+	+
INOCULATED PC	+	+
INOCULATED NC	+	+
CONTROL GROUP (GD4)	+	+
DRY HEAT (GD1)	-	-
AUTOCLAVE (GD2)	-	-
ETHYLENE OXIDE (GD3)	-	-
GAMMA RADIATION (GD5)	-	-

Table 1 – Sterility test - the signals "+" indicates the presence of microorganisms, and "-" absence, PC and NC are abbreviations of "Positive Control" and "Negative Control".

Histology images (Fig 4), using three different standards (Hematoxiline-Eosine (HE), Masson Goldner (MG) or Stevenel's Blue (SB)), evidenced a slight variation on cells' population mainly in HE and MG after exposition of sterilization procedures, being visible enamel (e), and dentine (d) in all pictures.

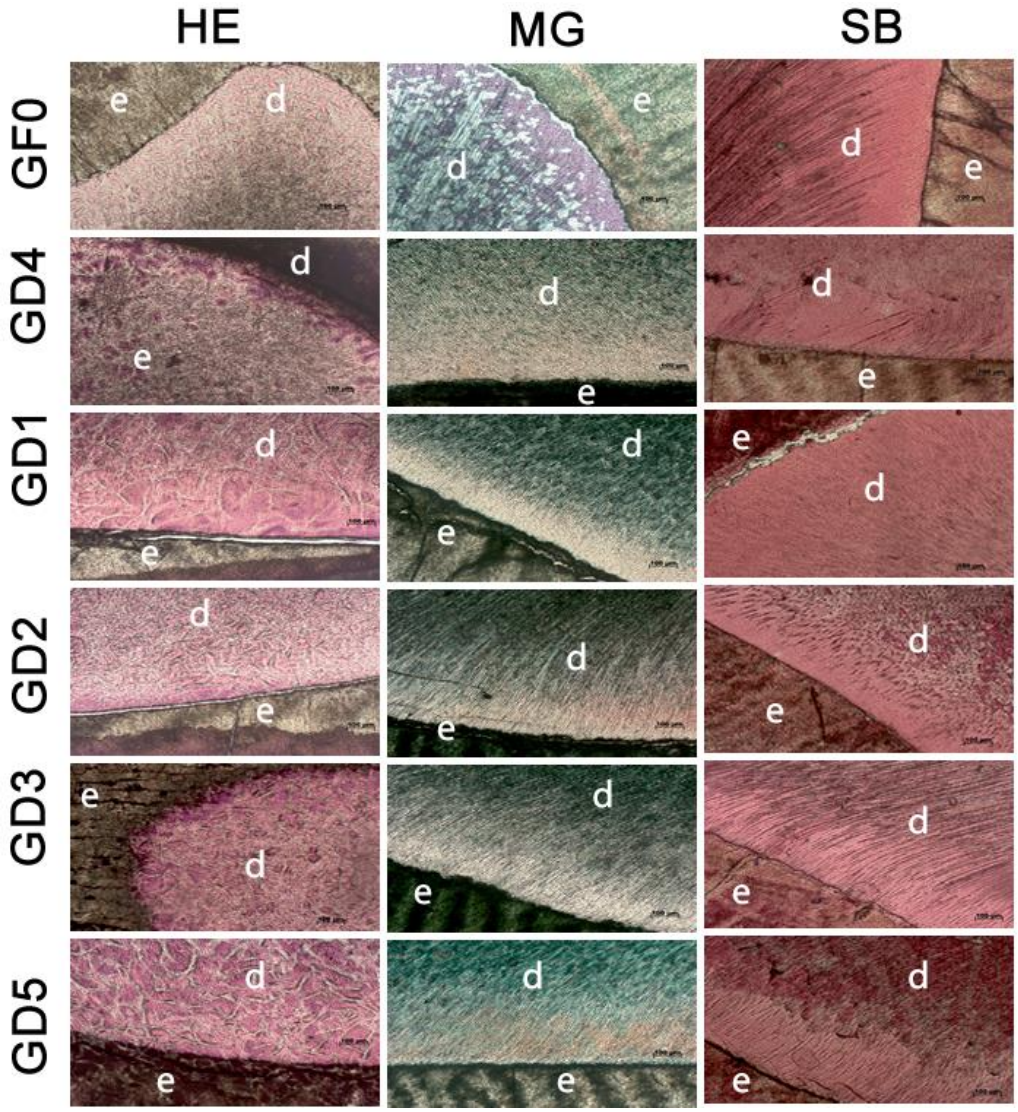

Fig 4 – Histology analysis – structural modifications were not identified, although a variation on population were noted specially on HE and MG, where “d” represents dentine, and “e” enamel region. Also was not identified any modification on the structure of the samples.

4. DISCUSSION

"The tooth’s design is a marvel of engineering, as it is capable to absorb static and dynamic energies"[31].The dental tissues are subject to compressive forces near 700N, shearing forces and large abrasive efforts[32,33]. Considered the most mineralized tissue of the human body, enamel is composed basically of crystalline PAH in Structure Ca (PO) (OH) and a low proportion of water and organic matter (protein and lipids).

The hardness of the enamel is between 3.2 and 4.4GPa or 270 to 360VHN and in relation to the fatigue strength is considered friable, usually presenting micro fractures by stresses beyond its elastic limit. Its tensile strength is close to 10MPa and its compressive strength is approximately 262MPa. Its modulus of elasticity in compression tests approaches 33.6GPa[32,34].

Dentin is a biological composite of a collagen matrix filled with nanometric apatite crystallites deficient in Ca (calcium), rich in carbonate, F (iron) and Mg (magnesium), and

scattered on parallel hollow cylinders called canaliculated or dentinal tubules[33]. The dentin has tensile strength is 50MPa and hardness from 50VHN to 60VHN[34].

The focus of analysis were exactly at the dentin region, where the control group presented hardness slightly below indicated by Garcia-Garcia (45.69, varying from 38 to 53HVN), Gamma Radiation presented the expected hardness (69.53, varying from 50 to 67HVN), Autoclave (69.53 HVN), Ethylene Oxide (70.61 HVN) and Dry Heat (77.55 HVN) presented final hardness significantly higher than indicated. It is necessary to confirm if this hardness increase can interfere on cell adhesion or not.

A comparison between control group (GF0) and the newly extracted tooth, on Scanning Electron Microscopy (SEM), were not identified significant structural differences between them. On the other hand, the decellularization process promoted a significant reduction of cells, justifying the removal of Formaldehyde Group 0 (GF0) from direct comparison, only were compared the results of Decellularized Group (GD1 to GD5).

After the sterilization treatment, no living microorganisms were detected in any sterilized groups on SEM images (Fig 1), agreeing with sterilization test from Brazilian Pharmacopeia [35]. And also were not detected alteration on the structural properties, except by a reduction of calcium concentration on samples and slight change coloration and micro-hardness.

At optical observation and colorimetric measures, were observed the color alteration in all groups treated with hydrogen peroxide (compatible with the quantitative measurements of colorimetric apparatus), but the macroscopic characteristics were kept the same.

The criteria of radio-opacity, shape and size were analyzed under the radiographic aspect, except by the formaldehyde group, all samples submitted to EDTA underwent demineralization and lost calcium, confirmed by XRF (X-Ray Fluorescence) analysis. No changes were detected on shape, size and anatomical radiographic criteria after the sterilization process.

At XRF analysis (Fig 3b) the larger weight losses (mainly calcium) was registered at GD3 (Ethylene Oxide Sterilization Group), while the XRD analysis (Fig 3a) indicated the crystalline structure of hydroxyl-apatite remained unchanged on the four sterilization groups. EDS (Energy Dispersive Scanning) analysis reinforced the hypothesis of no modification caused by the sterilization processes on ECM.

Comparing formaldehyde group (GF0) to decellularized groups (GD1 to GD5), at histology analysis (Fig 4) the significant reduction of visible cells provide evidences of decellularization process efficiency, and were not detected structural changes after sterilization processes. The efficacy of the four sterilization methods was confirmed by the Sterility Test.

5. CONCLUSION

This characterization methods confirmed no damages were caused to Extracellular Matrix (ECM) after any of the four sterilization methods (Dry Heat, Autoclave, Ethylene Oxide or Gamma Radiation.

In terms of sterilization, was confirmed residual pulp reminiscence after decellularization process, what, when exposed to THIO or TSB solutions, promoted a significant proliferation of

microorganisms, meaning some microorganisms were still active. The sterility tests also indicated the effectiveness of the four sterilization methods studied.

If considering a cost-benefit analysis, Dry Heat Sterilization and Autoclave methods would be the most indicated sterilization methods for dental biomaterial scaffold. It can be a feasible and promising alternative, however the effect of Dry Heat on dentine's hardness, or the color variations should be investigated to confirm the compatibility with stem cells previously to discard ethylene oxide or gamma radiation.

ACKNOWLEDGEMENTS:

To RADIONORTH - X-Ray images; UNIFESP (Federal University of São Paulo) Diadema campus through Prof. Dr. ROGERIO DE ALMEIDA VIEIRA and Prof. Dr. CRISTIANE ARRUDA RODRIGUES – Access to XRD, SEM-EDS, Micro-hardness equipment, and data analysis programs Topas & Origin; To FATEC-SP (Technology College of São Paulo) through Prof. Dr. DAVINSON MARIANO DA SILVA and Prof. Dr. SILVANO LEAL DOS SANTOS - XRF equipment; To IPEN–SP (Energetic and Nuclear Research Institute – 60CO Gamma Rays equipment; ACECIL – Ethylene Oxide equipment; To USP (University of São Paulo) through PROF. Dr. DÉBORA DE OLIVEIRA and PROF. Dr. FELIPE REBELLO LOURENÇO – Sterility test equipment.Government Funding agency CAPES (Coordination of Improvement for Graduated Level Personnel).

AUTHORS CONTRIBUTIONS:

Author 1 (Iwamoto, L): Contributed to conception, design, data acquisition, analysis and interpretation, performed all statistical analysis, drafted, critically revised the manuscript and gave final approval;

Author 2 (Duailibi, M.): Contributed to conception, design, analysis and interpretation, performed all statistical analysis, drafted, critically revised the manuscript and gave final approval;

Author 3 (Iwamoto, G.): Contributed to conception, design, data acquisition, analysis, interpretation and critically revised the manuscript and gave final approval;

Author 4 (Oliveira, D.): Contributed to design, data acquisition, analysis, interpretation and critically revised the manuscript and gave final approval;

Author 5 (Duailibi, S.): Contributed to conception, design, analysis, interpretation, critically revised the manuscript and gave final approval;

* "All authors gave their final approval and agree to be accountable for all aspects of the work."

CONFLICT OF INTEREST STATEMENT:

The authors explicitly state that there are no conflict of interest.

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