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X Ray Diffraction: An Approach to Structural Quality of Biological Preserved Tissues in Tissue Banks

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1. Introduction

The purpose of this chapter is to introduce new methods of analysis, to evaluate the final quality of human origin bio therapeutics products generated in Tissue Banks (TB), using well developed and known techniques in various fields of Physics, Chemistry and Biology as applied X – Ray diffraction (XRD), and Raman Scattering (RS).

Cryopreservation techniques are fundamental supports in the conservation procedures of biological materials in TB work. However, controversial views remain on the effects at the molecular level that cryogenic temperatures and thawing could produce on the functional structures of tissues. The same concept can be sustained to glycerolized tissue preservation. Taking into account this scope, we implemented a methodological scheme to analyze tissue specimens before and after programmed cryopreservation, or glycerolization in order to find structural differences in the basic material constitutive collagen, using the techniques formerly mentioned: diffractive and scattering.

It is noteworthy that both methods of analysis can be applied to any type of tissue preserved for the aforesaid purposes, with other conservation techniques, such as freeze drying or un programmed freezing.

2. The tissue banks and the "viability" of it's therapeutically products

The TB are technical establishments whose main institutional objectives are collection, preservation, storage, release and distribution of biological tissues for therapeutic use in transplantation medicine. These objectives is met according to scientific criteria from agreed international protocols (Spanish Association of Tissue Banks: AEBT, International Atomic Energy Agency, IAEA, European Association of Tissue Banks: EATB; American Association of Tissue Banks: AATB) and according to the legal frameworks of the different countries and

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their ethical rules. On the other hand, tissue banking activities are carried out following strict bio-security criteria by the selection of suitable donors, permanent quality control and continuous management of physical plant, equipment, supplies procurement procedures, and end products, which must comply criteria for "viability" therapeutic apply.

According to (Pegg 2006) this concept of "viability," applies whenever a graft of tissue obtained as final product, meets the leading natural-biological function for which it was preserved, and that is pathologically affected in the recipient. This explains why there are different procedures and methods of conservation in TB, according to the type of tissues and its expected restored function.

In some cases, we must preserve, as the main function, a biological synthesis, which requires mandatory of vitality in cellular functions (eg, parathyroid glands, or ovarian tissue). In other cases, the objective is to preserve static and mechanical functions as a segmental allograft bone support, or biodynamic behavior, as in cases of preservation of blood vessels. This implies that "viability" is not necessarily synonymous with "cell vitality" and therefore the requirement is to preserve elements of the Extra Cellular Matrix (ECM).

Hence, the importance of assessing possible changes on the components of the ECM that TB procedures may be generate on processing and stocking biological tissues.

3. The concept of functional structure of ECM

The ECM was considered early in the twentieth century as filler material and mechanical support of cell structures, which was thought as the only protagonists of tissues functionality. In the 50´s, (Grobstein, 1953) proposed that the induction in the development of a tissue depended on the presence of ECM.

In the 60´s, (Hauschka & Königsberg, 1966) establish that pig embryonic muscle cell cultures proliferate more properly in a media with presence of metabolic products from fibroblasts, and identify the collagen to induce the development of them. This protagonist role of collagen in the processes of induction and cell proliferation, is corroborated by (Meier & There, 1974), by testing the inductive capacity of collagen, for the synthesis of ECM in the corneal epithelium of pig embryos (5 days old development). (Sanders 1988) working on neural crest and sclerotome cells of early chicken cells embryos, prove that the presence of type I collagen is necessary for cellular migration and *de novo* synthesis of ECM is a prerequisite for normal cell migration and attachment in earliest stages of embryogenesis.

Additionaly, since the early 80´s (Bissell 1982), work in the field of cancer biology emphasizes the importance of "micro" immediate cellular environment and posits the hypothesis of 'Dynamic Reciprocity' by which the ECM contact trans membrane receptors, influence gene expression through signals transmitted via cytoskeleton, generating so "new" products for the ECM. Thus, the cell and the ECM, form a binomial reciprocal exchange interaction, which has vital importance in the early stages of embryonic morphogenesis and later in postnatal life, the physiological mechanisms of growth and development as well as in response to injury. (Bissell, 1982; Davis, 2010; Kelleher, 2004; Nadiarnykh, 2010; Schultz, 2005; Schwinn, 2010). The ECM consists of a complex variety of macro molecules that can be summarized schematically as follows: 1) protein collagen, 2) structural glycoproteins, 3) proteoglycans and glucosamine glycans, and 4) elastin. These complex and diverse

molecular groups, organized into super families, are shown with a dynamic distribution, and functional modulated behavior, with variations between different tissues. This bio plasticity is observed even within the same tissue type, as homeostatic biochemical, and bio mechanical requirements, including interactions with various molecules: growth factors, cytokines, enzymes and other inducers synthesis products as well as lytic and degradative matrix one.

The surface receptors of the cell membrane, in close contact with this complex and dynamic molecular ECM set, interact through the cytoskeleton to the genome, which modulates the different stages of ontogeny, growth and postnatal development sequences as well as molecular structural and functional physiology and patho physiology biological requirements of tissues. (Abraham, 2007; Bowers, 2010; Worthley, 2010). Related to the own collagen structure there are ligands and functional domains that take contact with other ECM molecules (fibronectin, proteoglicans, and collagen - collagen interactions) and with the neighbor cells microenvironment (cell integrin receptors). Several poly peptid sequences, (eg.: GFPGER: glycine - phenylalanine - hyroxyproline - glycine - glutamic acid - arginine) and ligands domains (eg.: Matrix Metalloproteinase Interaction Domain or MMP ID; Colagen V Cross-link site or Col V X-link), have been identified and play an important role in regulation of migration, proliferation, adhesion and apoptosis in biology cell tissues. (Orgel, 2011; Sottile, 2007; Sweeney, 2008).

It is obvious therefore that the extra cellular medium, forms a molecular complex of plastic, in both dynamic up regulator as down regulator in constant cross talk with cell pole whose points of contact and mutual information imply the presence of binding sites at ECM structure related to cell surface receptors. Fibril collagen constitutes approximately 25% of tissues for all species of mammals and is the main component of the total molecules that make up the ECM. (Kielty, & Grant, 2002). To date, they have been described up to 29 different types of collagens with the corresponding genetic determinants. "Structural" Collagens in the ECM are called fibrils (Fibril Collagen) consisting of types I, II and III, V and XI. Type I constitute 90% of body collagen and mainly, perform mechanical resistance functions. In addition it provides three - dimensional modeling formation of tissues. An important bio molecular feature of our study is it hierarchical and sequenced shaping showing collagen. Taking collagen I model, pro collagen, amino acid primary structure have a intracellular synthesis (endoplasmic reticulum), with repeated tripeptides, design whose residues are Gly-Pro- Hyp or Gly-X- Hyp which Pro and Hyp are near to 17% and 33% respectively. Therefore 50% of the average 1000 residues of the total composition of the molecule, pro collagen, are other amino acids. Its length of 300 nm and width of 1.5 nm, is organized in a left-handed secondary structure of three amino acids per turn, with Gly residues central and peripheral Pro and Hyp out of the spiral. Three assembly helical pro collagen monomers (2 α1 and 1 α2) in right-handed configuration, determine the tropo collagen structure in the extra cellular space. In this space the molecule is arranged in staggered bundles with a gap of 67 nm by inter molecular bonds tropo collagen units, which gives to the collagen fibril new product design, a repetitive sequence which observed in the ME identified a characteristic D - banding. The final design shows collagen fibers arranged, spatially distributed in regular packages along the lines of force of the biomechanical characteristics of each tissue. This last aspect brings an added dimension of ordering design given by the spatial distribution of fiber bundles, and their inter reciprocal space.

These three characteristics: a) repetitive and periodic sequencing of the D - banding, b) structuring hierarchically ordered by supra molecular complexes, and c) nano-scale dimensions of the structures, made of collagen complex an para crystalline super molecule, liable to be analyzed by techniques diffractive as discussed later. (Sweeney, 2008; Berenger, 2009).

In this context becomes important the analysis of the changes that for preservation purposes, can be induced in the molecular components of the ECM. Particularly taking account that collagen is the main structural component of the extra cellular microenvironment and has a proven role in the functional biological mechanisms, developmental, physiological homeostatic and physio - pathological tissues behavior. (Kielty & Grant, 2002; Orgel, 2011).

These reasons justify work TB, to design tissue preservation processing models, with conservation of the collagen component, from both, structural and biochemical characteristics. It must be take in mind, that allografts should meet a homeostatic interaction with cell biology recipient patient, through its membrane receptors in functional contacts with molecular ligands and domains of preserved ECM, to improve physio pathological situations.

Note therefore that the biological behavior of a suitable allograft depend on the presence and indemnity of molecular epitopes or ligands, which can be eventually altered in its stereo chemical distribution during cryogenic or glycerolized procedures.

4. The interaction between ECM components, and the physic-chemical phenomena preservation procedures

About the cryogenic effects on ECM, there are several references in different disciplines, about the ultra cold temperatures on biological material. Indeed, at the molecular level have been observed different types alterations generated by freezing / thawing phenomenon, on biological structures. As earlier in the 60´s, (Levit, 1962; 1966), had postulated irreversible changes in the tertiary structures of soluble vegetal proteins, with loss of its biological capacity, such as the rearrangement of disulfidric functional bonds to non-functional disulfide covalent configurations.

The concept of "repulsion hydration forces" refers to cell membranes, was developed by Wolfe, J. (1999). These phenomena is induced by water efflux through such semi-permeable membranes during cooling process, promoting large mechanical stress and strain in the biological structures, and generating physical deformations and changes in the molecular functional membrane behavior. Such condition is done under the observed ground of the structure of the crystalline ice mass in the extra cellular space. This, results in intra cellular dehydration of the tissue and the extra cellular hyper osmolarity of super cooling liquid. So, it generates a displacement of inter atomic and molecular chemical equilibrium that, explains changes in the stereochemistry and molecular architecture of biological structures. This scope would agree to Levit postulates. (Levit, 1962; 1966).

The effect that the conventional criopreservation exerts on the ECM structure is controversial information. (Gerson, 2009), comparing morpho structural collagen mesh from

fresh and cryopreserved human heart valves by second harmonic generation, sets no changes between both categories. However, it was found extensive damage in collagen structure in porcine frozen leaflets related to fresh control one, using laser-induced auto fluorescence imaging , (Schenke Layland, 2006), and second-harmonic generation. (Schenke Layland, 2007).

In other field, there are many studies showing that the biomechanical behavior of tissue collagen framework, is not altered by effect of cryopreservation / thaw cycle, in vascular (Armentano, 2006; Bia, 2006; Langerak, 2001, 2007; Pukacki, 2000**),** tendon , (Woo, 1986; Park, 2009) or bone tissues. (Hamer, 1996).

However, controversial literature is also observed for biomechanical variables. (Rosset, 1996) observed in vitro, decreased compliance and hysteresis an increase of modulus of elasticity in thawed cryopreserved human carotid arteries, related to fresh one. (Gianni, 2008) found that the freezing of human posterior tibial tendons significantly affected behavior in vitro biomechanical performance. Finally, either way, under many point of view is possible highlight that the functional character of fibril collagen depending to the particular structural and biochemical preservation, which may be damaged during cryopreservation defrosted process in TB.

About the interaction between alcohols and polymerized amino acid, in early 70´s (Frushour, & Koenig, 1975) postulated, in Raman Scattering field, that methanol modified an aqueous poly-DL-alanine (PDLA) solution, by disruptions of the helical regions by breaking the hydrophobic bonds.

5. Diffractometry: a tool for analysis of structural ordering of collagen

5.1 The matter and its organization

It defines that the spatial arrangement of ions and atoms of matter, have a crystalline profile when its design shows a repeating sequence. The frequency of repeated and symmetrical distribution of the atomic stereo chemical units constituents of matter, determine the solid crystalline character. It is understood that a substance is “homogeneous” when each constituent unit of the solid is linked by chemical bonds to another identical unit in any sense of space, and is identified as the ideal “homogeneous crystal” model when, theoretically, is infinitely extended in space. The sequential nature of the repetitive and symmetric atomic elements, defines the spatial network model, under the so called “cells ordering”. The three dimensional symmetric distribution of elemental units of the complex let likened to an orderly succession of planes separated by a distance "d". This design is easily identifiable in crystalline substances of inorganic chemistry: quartz, diamond, graphite, etc. Diffracted analysis of inorganic or organic crystalline matter can provide detailed information about molecular design, related to intermolecular distance and stereo chemical angle conformation. In the world of bio molecular chemistry, matter is organized by more complex models, through an extensive variety of atomic molecular combined structures. In this picture certain combinations become repetitive units, consisting of several basic types of atoms links together by different kinds of bonds. Nevertheless, one can observe the character of certain spatially ordered molecular configurations, which are equally repetitive. This setting defines the so called “molecular crystals”, despite not

showing the perfection system of "homogeneous crystals". So, no bond lengths and angular atomic positions can be determined, but an approximate view about relative ordering structure is given applying XRD techniques. These structures are typical of biological substances such as proteins or DNA molecules whose functional design depends on the molecular arrangement, and type of chemical bond established between its molecules.

5.2 The diffractive phenomenon and elastic scattering of x-rays

X-rays are a form of electromagnetic energy produced by a source to be impacted by electrons of high kinetic energy supplied (usually a tungsten filament named cathode). The incident electron impact, destabilizes the internal atomic orbital of a target material (an anode built with pure copper), generating atoms in electronically excited state. The movement of electrons from outer orbital to balance the impact generates heat energy and emission of x-rays, in a spectrum of wavelengths (λ) measurable in Angstrom units (**1 Å = 10^{-10} m**). The band spectral x-rays emission is "filtered" through mono chromator to obtain a single wavelength that corresponds to the "characteristic radiation" used in the x-rays crystallography: K-alpha line (Kα) for each material property anode of the device. To our work, XRD is defined by the interference between monochromatic characteristic emissions (Kα) with the ordered material in crystalline form. When a photon interferes with an orderly molecular structure without loss of energy (elastic scattering), produces a deviation from its original direction which is the diffraction phenomenon. The condition for diffraction to be possible is that the distance between the periodic and ordered structure elements (atoms or molecules), fall in the wavelength range of incident ray. The condition to be detectable is that some degree of ordering is present in the material to be studied, in order that the interference could be constructive. As previously mentioned, collagen shows particular characteristics like a biomolecular arrangement built by: a) the hierarchical supra molecular order fibers, b) the observed crystalline structure of the spaces sequenced "D" of fibrillar collagen, c) the nano scale distance of repetitive molecular units, all of them allows to analyze biological stroma collagen by the XRD. (Aspden, 1987; Berenguer, 2009; Connon, 2007; Hickey & Hukins, 1980; Horton, 1958; Pauling & Corey, 1951; Pérez Campos, 2008).

The equation that allows the practical application of this technique is Bragg's Law.

$$n\lambda = 2d \sin\theta \quad (1)$$

Formula 1 Bragg´s Law**: n** is an integer**, λ** is the x – ray monochromatic wavelength, d is de distance between the planes of the crystalline net, and **θ** is the angular value between the incidental x –ray and the considered crystalline plane**.**

Given a certain incidence angle of a monochromatic beam on the material structure, sequencing crystalline spacing "d" determines a dispersive interference when the rays are emerging in construction phase. Emerging rays can be recorded in a Cartesian coordinate system where the independent variable "x" records the range of 2θ values and the dependent variable "y", the relative values (R.V.) of the ordering lattice system. The recordable diffraction graphics or diffractogram depend of the content and the atomic distribution within the repetitive units that define the three dimensional arrangement. (See Figure 1)

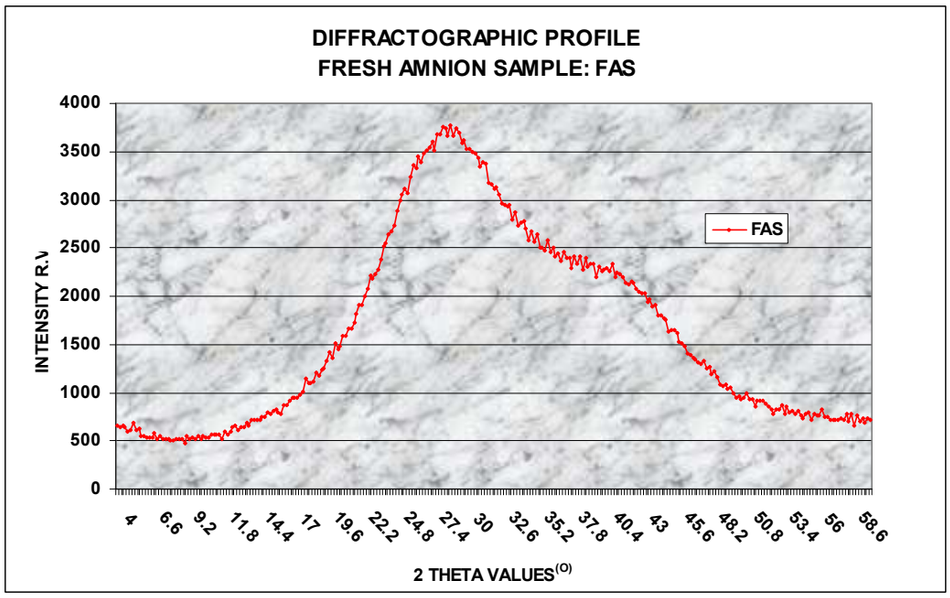

Fig. 1.

5.3 The Raman effect, or inelastic scattering of light

Given a monochromatic light beam incident on a material, there will be a phenomenon of elastic light scattering as a result of the interaction of photons and electron atomic elements of the network links. The elastic scattering implies that the frequency of the incident light beam and the scattered light emerging is the same, so that no changes have occurred in the respective energy levels. This phenomenon known as Rayleigh scattering is highly significant from a statistical point of view. However an extremely low intensity incident light (in the order of 1 photon in 10^7 to 10^{10}) shows inelastic behavior in the interaction with the structure determining slight changes in the emerging wave frequency, which depends on the characteristics of matter incised. This phenomenon or Raman Effect was discovered by (Raman & Krishnan, 1928), and allows the chemical structure analysis of biological material.

Atomic particles mass and its energy states (vibration and/or rotational), maintain chemical bonds that define crystal structure, sustaining dynamic design stability by neighbor interaction. The movements of both vibration and rotation of the particulates: (υ = frequency), defined the dynamically stable energy level where they are. If the interaction of a beam of photons of frequency (υ_0), print a change of unstable frequency at the particle network link, they scatter photons with a different frequency (υ_r), define an inelastic scattering. The energy can be dispersed in a model $\upsilon_r > \upsilon_0$ called scattering Raman - Stokes or $\upsilon r < \upsilon_0$ know as scattering Raman anti Stokes. υ_r values are characteristic of each design structures and define atomic molecular matter. Approximately 99% of the output assays is Stokes Raman scattering hence, those are the models profiles recorded. In the Cartesian co ordinate system the independent variable x records the difference $\boldsymbol{\upsilon_r - \upsilon_0}$ in cm^{-1}, and the ordinates y, the scattering intensities for each differential rate, in relative units (R.U.) (See Figure 2)

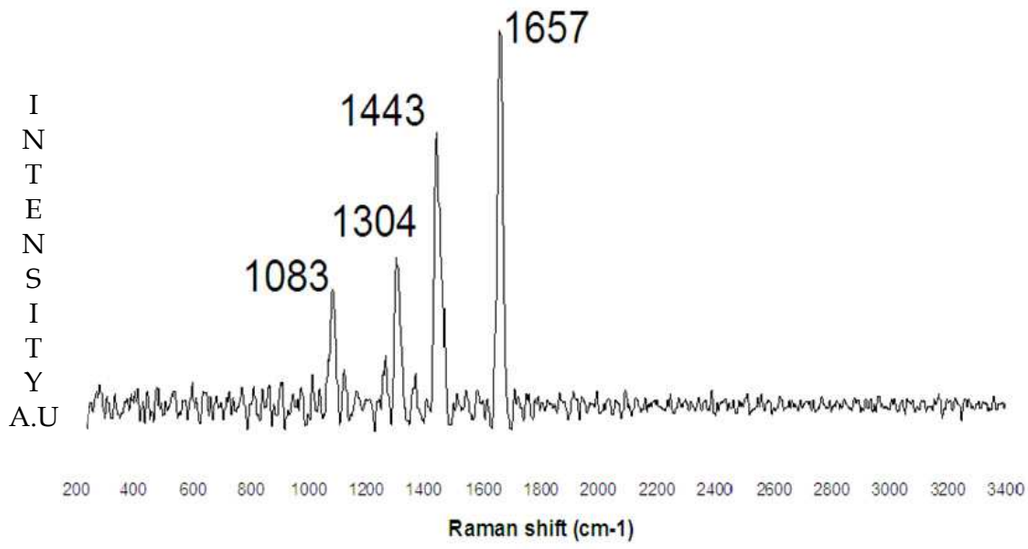

Fig. 2. Raman Spectum oleic acid

5.4 Interaction x-rays - Collagen: A model analysis

The x-rays diffractive analysis of the collagen structure was earlier studied (Pauling & Corey, 1951). Defined diffraction peak was described, in the range d = 2.86 Å (2 θ = 31.3 º using CuK α radiation λ = 1.5418 Å value), based on the criteria of the Bragg Law. This phenomenon was interpreted as the expression of cis configurations for the amide groups of the polypeptide chain.

Using this background, our group analyzed tissue banking allograft, in order to compare the diffraction profiles obtained before and after cryo-preservation method (vascular tissues and amnion tissue), and glycerolized preserved method (amniotic membrane). The working hypothesis states that the preservation methods can modify the stereochemistry molecular structures, determining changes in collagen and the consequent differentiation of diffractive or dispersive profile.

6. Materials and methods

6.1 Donors and biological samples procedures

The applied procurement protocol to vascular tissues was made on cadaver multi organ donors through informed consent and in accordance with standard operating manuals in the National Institute for Donation and Transplantation (INDT) of Uruguay. They were likewise applied legal and ethics regulations (Law 14005/1971 - 17668/2003) valid in our country. The exclusion criteria and biological safety were applied, in accordance with the Standards for Tissue Banking: International Atomic EnergyAgency (IAEA - 2005) and the

Spanish Association of Tissue Banks (AEBT - 2005). The same selection criteria, exclusion, tissue procurement and processing were applied to living donor placenta with clinical controlled normal pregnancy and delivery, by the parameters set by the Ministry of Public Health of Uruguay.

Donors were selected according to the protocol in a range between 18 and 60 years (35.5 ± 11.8 years mean age, 47% M 53% F) obtained by aseptic dissection, 10 aortic arterial segments, and 8 carotid. It proceeded under a laminar flow cabinet to cleaning, package, and storage in physiological saline at 4°C. Fresh Vascular Samples (FVS) were shipped to DETEMA within 24 hs, for XRD. The same process aseptic protocol was applied to 6 amnion obtained by manual dissection from donor placenta. Fresh Amnion Samples, (FAS) were stored in saline solution at 4ºC and shipping to DETEMA within 24 hs for XRD analysis.

The Cryopreserved Vascular Samples (CVS), segments of each contra lateral carotid donor, and hemi ring segments of thoracic descendent aorta, were processed for cryopreservation in a Controlled Rate Freezing System (Model 9000, Gordinier Electronics, Inc. Michigan). Stored CVS were maintained up to 30 days at -142ºC. The cryopreservation media was: RPMI 1640, 85 cc; Human Albumin (20%), 5 cc; DMSO 10%. Cryopreservation was made into termal sealed double cryo resistant bag (Joisten and Kettenbaum D51429, Bereisch Gladbach, Mod.011342). The mean cooling rate applied was -1ºC/min from 4ºC to -90ºC and then quickly stored at -142ºC during 30 days in steam liquid nitrogen.

Same procedures were applied to obtain 2 Cryopreserved Amnion Samples (CAS), stored up to 30 days at -142ºC.

Defrost protocol applied for vascular and amniotic tissues, was according with Pegg et al. (1997), and defrosted samples were shipped to DETEMA for XRD.

6 Glycerolized Amnion Samples (GAS) were obtained by soaked in screw cap flask in Glycerol (95%) and stored at 4º C for 30 days. Glycerol from amnion was removed by three sequential shaking washing for 15 min. each, in saline solution and then shipped to DETEMA for x-rays diffraction, and Raman Scattering analysis. The comparative assays were done with FVS vs CVS; FAS vs CAS; and FAS vs GAS.

6.2 Diffractographic and Raman scattering technical procedures

XRD measurements were conducted at the Laboratory of Crystallography, Solid State and Materials, School of Chemistry (DETEMA), with a CuKα radiation source of wavelength λ = 1.5418 Å, using a Rigaku Ultima IV diffraction system. The incident ray is calibrated to arterial vessels in a range for 2θ between 5 ° and 60 °, step scan of 0.1 ° for 10 sec. each. The respective diffraction profiles (FVS vs. CVS) were filed for later analysis. Comparative profiles for amnion (FAS vs. GAS; and FAS vs CAS) were treated the same way as having been calibrated for 2θ between 5º and 60 º ranges scanning with steps of 0.2° for 10 sec each.

Raman spectra were recorded using a Raman DeltaNu Advance 532 spectrometer with a laser frequency doubled Nd: YAG, 100mW, with a 532 nm wavelength, scanning in the 200 and 3400 cm^{-1} region.

6.3 Planimetric analysis: Obtaining the order coefficients for XRD (Perez Campos, 2008)

Given the diffraction profiles of two tissues A and B to compare tests, we can define the respective planimetric surfaces, defined under the corresponding diffraction curve, that are a function of the degree of molecular arrangement of the studied tissue.

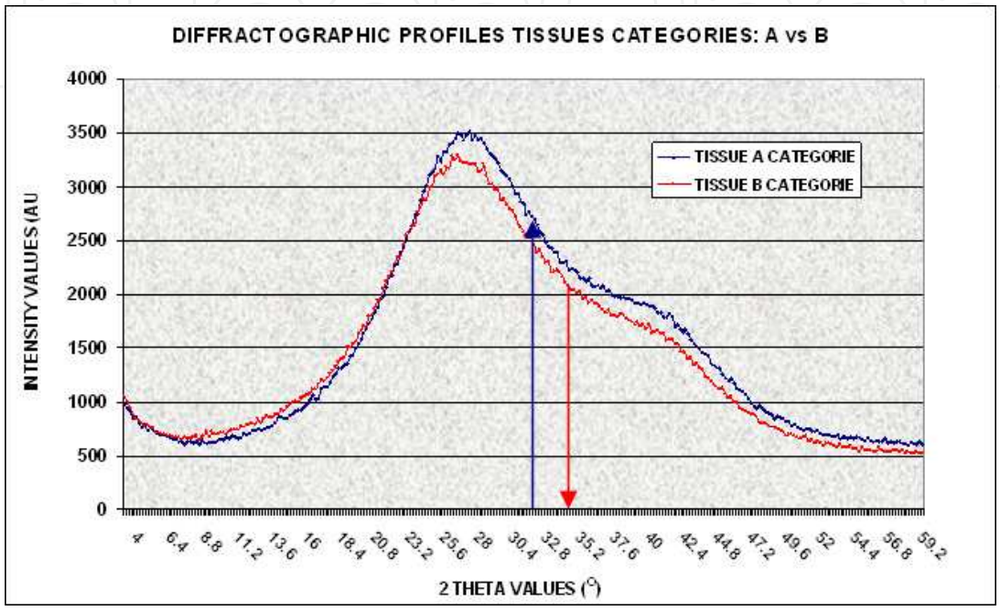

Fig. 3. Diffractive profiles from two different tissues categories A vs B.

Relative Differential Intensity values (RDIV) established for each 2 θ point between 5º and 60º, will produce a result that: if absolute value tissue A > tissue B will have a resulting positive (+) value; but if tissue A < tissue B will have a resulting negative (-) value.

With those values obtained in each point from 2θ it can be developed Differential Planimetric Surfaces (DPS) that represent the sum of every relative intensity value.

Its mathematical expression is given by the equation:

$$\Sigma \left(\uparrow - \downarrow \right) \times I_{A-B} [2\theta\ (^{o})] = DPS \qquad (2)$$

Where (↑) represents ordering diffractometric intensity for ***y*** axes values of tissue A in one point in 2θ; (↓) represents ordering diffractometric intensity for ***y*** axes values of tissue B, in the same point in 2 θ; I_{A-B} is the difference between each respective ordering diffractometric intensity for ***y*** axes value at the same point in 2θ. Finally, [2θ (º)] is each point in **x** axes between 5º and 60º angular incidence.

DPS can be edited in a Cartesian model too, where *x* axis is 2θ values and *y* axes is the Intensity Relative Differential Values (IRDV) between both comparative samples for each point 2θ values. (See figure 4)

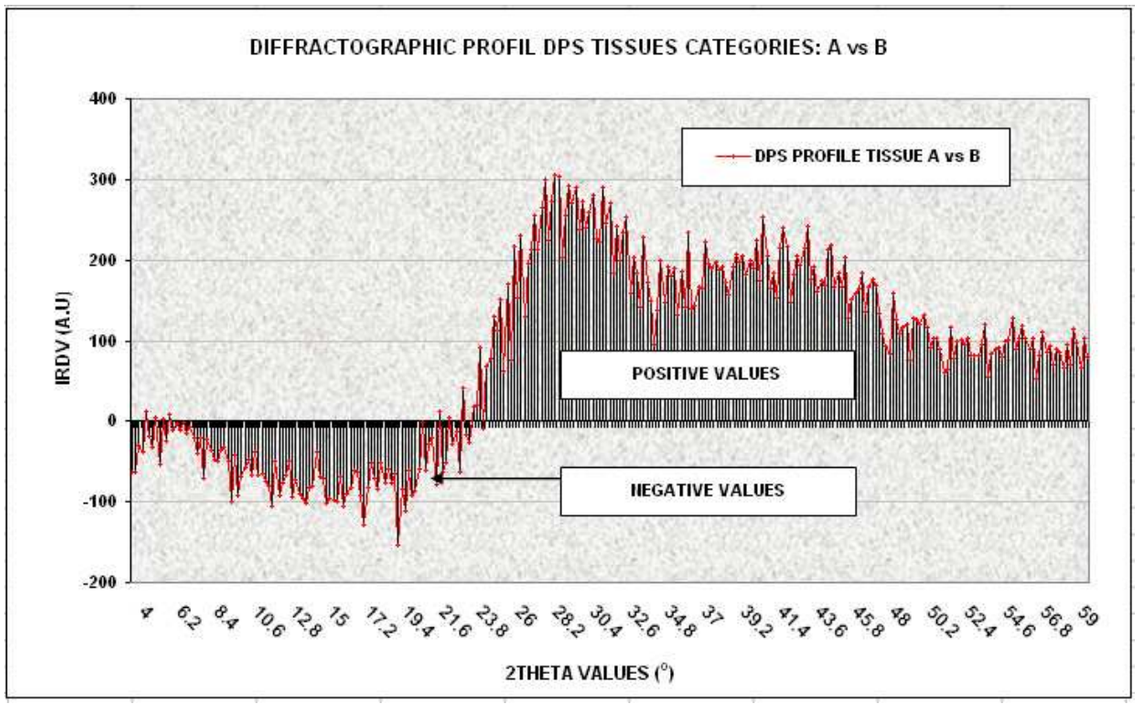

Fig. 4. DPS from two tissues categories A vs B to be studied by X-ray diffraction.

The ratio DPS (+) values vs DPS (-) values define the Ordering Profile Coefficient (OPC) according to the following formula:

$$OPC = \frac{DPS + VALUES}{DPS - VALUES} \tag{3}$$

OPC absolute values are always above 0 and they are greater than 1 when +DPS values > -DPS values. When +DPS values < -DPS, OPC falls into an interval greater than 0 and lower of 1.

7. Results

7.1 Cryopreserved vascular tissues results

Analysis of the diffraction curves shows that regardless of the condition FVS or CVS, the same design with a peak of maximum intensity to 31.3 º and another lower, at 42 ° in 2θ is kept, whether there are noticeable differences in design profiles between the two categories, even for a single donor. (Perez Campos, 2008). Comparatives diffraction profiles shows the confirmation of a peak intensity for 2θ = 31.3 ° corresponding a d - spacing = 2.86 Å. The lower peak intensity, obtain d – spacing = 2.15 Å applying Bragg Low calculus. This behavior is independent of the vessel (aorta or carotid) and the processed sample (FVS or CVS). See Figure 5

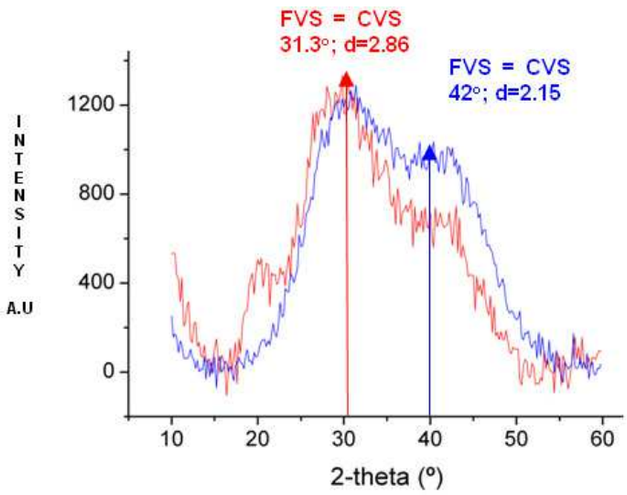

Fig. 5. Diffractografic profiles from thoracic descending aorta. Code color: FVS, Red; CVS, Blue. Note: The 1st and 2nd maximum peak labels of each respective diffractive curve indicate; Category of tissue: 2 Theta value; and calculated d spacing.

Calculated OPC values in respective analyzed vessels, shows a greater crystalline framework for CVS vs FVS, regardless the kind of arterial segment: aorta or carotid. 75% of aortic samples showed OPC values > 1 and 62,5% of carotid samples had the same behavior. Perez Campos et al (2008). Figure 6 show DPS defined from diffractografic FVS and CVS of Figure 5:

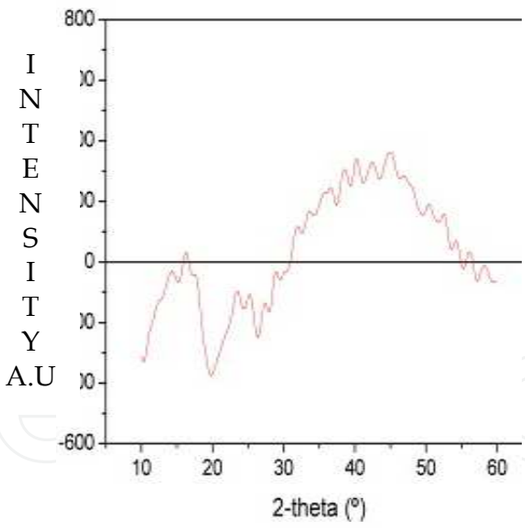

Fig. 6. DPS profile FVS vs CVS in a descending thoracic aorta from a male donor 50 years old.

Note the great difference of design shape of DPS curve related to the same one obtained from amnion tissues; (see below).

7.2 Glycerolized amnion tissues results

The diffraction curves of the glycerolized amniotic membrane, also shows the same kind of form and design for both the FAS and GAS. Notwithstanding the maximum diffractive peak

in FAS d spacing = 3.24 (28.4 ° in 2θ) and in GAS, d spacing = 3.28 (28 ° in 2θ). A second peak is shown to both kinds of samples FAS and GAS for same d spacing = 2.35 (40.4º in 2θ). Contrary to the notable profiles differences showing in the two categories of vascular tissues (FVS and CVS), both amnion profiles -fresh and glycerolized- have almost the same design curve. (See Figure 7)

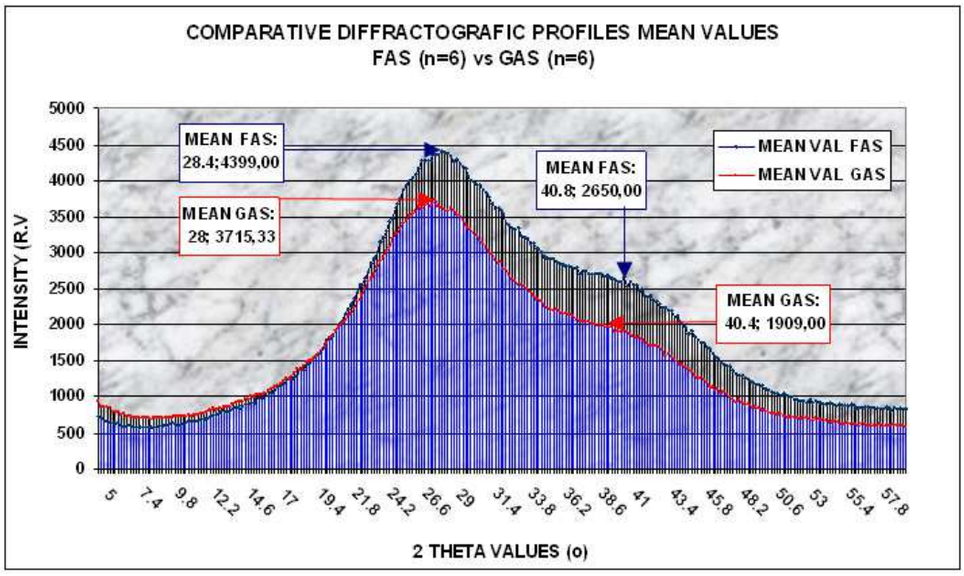

Fig. 7. Diffractive curves FAS and GAS profiles. Note: The 1st and 2nd maximun peak labels of each respective diffractive curve indicate; Categories of tissue: 2 Theta value; and Intensity RV.

Mean diffractographic profiles for 6 FAS vs 6 GAS let us obtain DPS picture and calculate OPC values = 14.76 (See respective Figure: 7 and Table: 1)

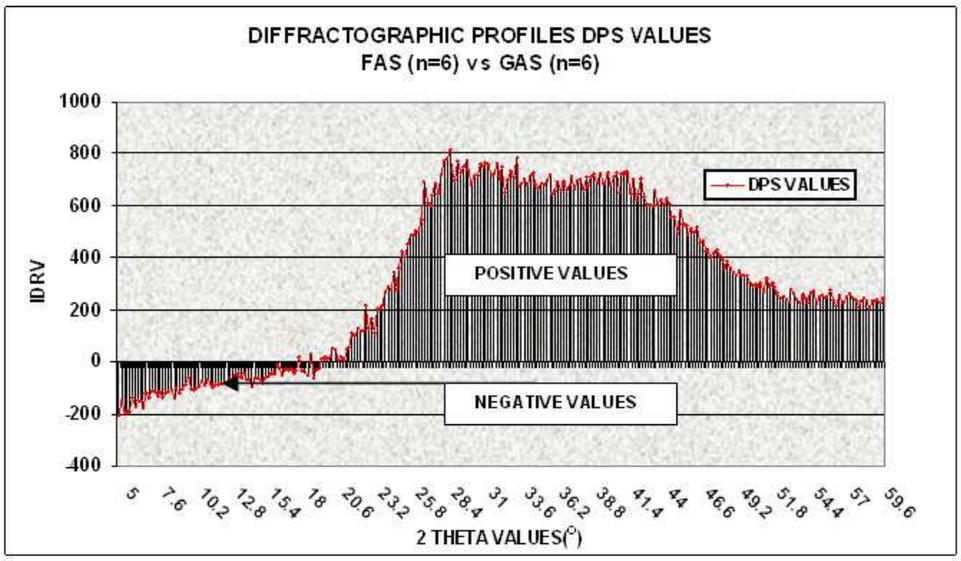

Fig. 8. DPS profile by FAS vs GAS analysis obtained.

OPC CALCULATION FAS vs GAS		
OPERATION		ABS. VALUES
∑ DPS + VALUES		94228,17
∑ DPS - VALUES		-6385,33
OPC VALUE = (+DPS) / (-DPS) = 14,76		

Table 1. OPC value from FAS vs GAS.

7.3 Cryopreserved amnion tissues results: X-ray diffraction

4 FAS vs 2 CAS was analysed. The same phenomena of Glycerolized amnion about form and design maintenance, was observed between FAS and CAS. Equally, there are a maximum peak in FAS d spacing = 3.26 (28.2º in 2θ) and in CAS, the same d spacing. Also a second peak is detectable to FAS in d spacing = 2.36 (40.8º in 2θ) and to CAS with equal values. (See Fig 9)

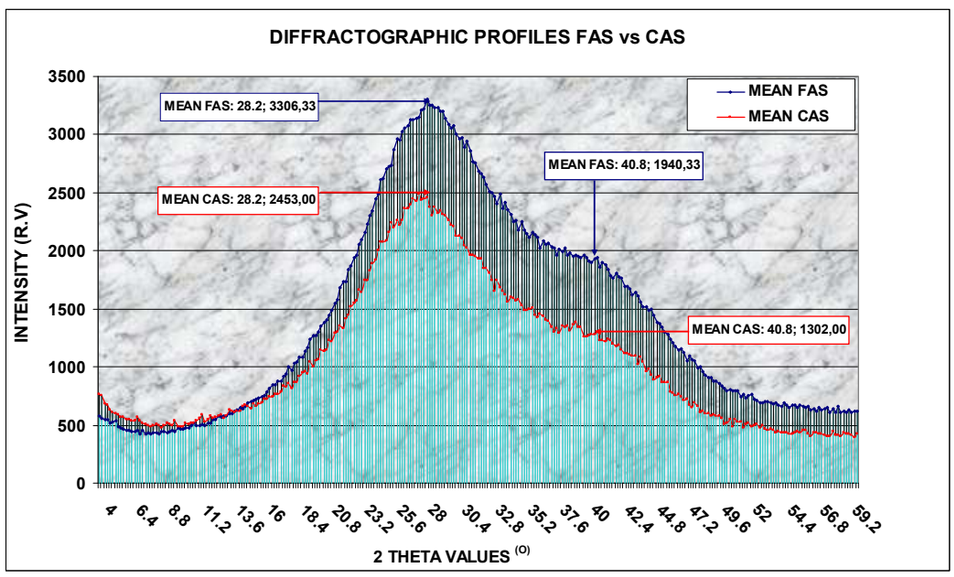

Fig. 9. Diffractive curves FAS and GAS profiles. Note: The 1st and 2nd maximun peak labels of each respective diffractive curve indicate; Categories of tissue: 2 Theta value; and Intensity RV.

Figure 10: show DPS profile obtained from operative subtractions analysis between FAS Vs CAS respective diffractive curves:

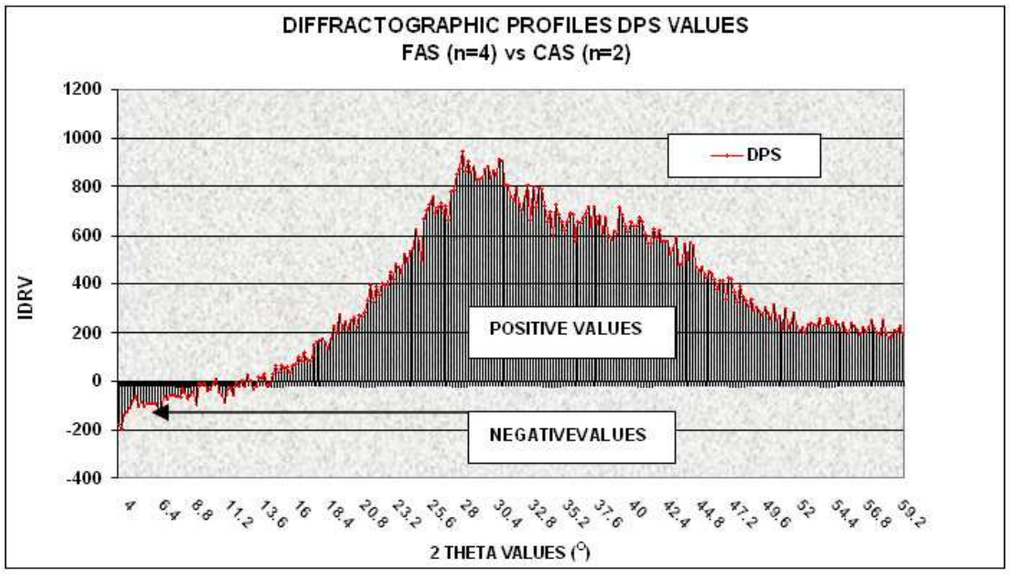

Fig. 10. DPS planimetric picture FAS vs CAS.

Operative planimetric values DPS obtained showed an OPC = 42.02 (See Table 2).

Newly highlight the notable differences in shape and form between vascular and amnion DPS pictures.

OPC CALCULATION FAS vs GAS		
OPERATION		ABS. VALUES
∑ DPS + VALUES		102887.67
∑ DPS - VALUES		-2448,33
OPC VALUE = (+DPS) / (-DPS) = 42.02		

Table 2. OPC values FAS vs GAS

7.4 Cryopreserved amnion tissues results: Raman Spectra

Figure 11 Show Raman Spectra profile from FAS vs CAS assays. Note that having regard to the best imaging definition, and taking account the meaningful change area of Raman Shift between both categories, the showed range is 1000 – 2000 cm^{-1} in x.

It should be highlighted that arrows points marked correspond to noticeable differences in positive Intensity values (AU) between FAS (red line) and CAS (blue line).

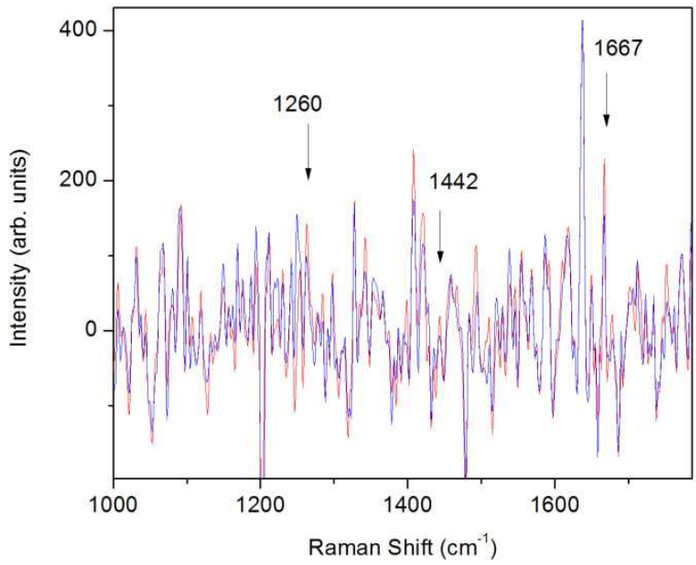

Fig. 11. Raman Spectra FAS vs CAS to area 1000 – 1800 Raman Shift (cm^{-1}). Color code; Red: FAS, Blue: CAS.

8. Comments

8.1 X-ray diffraction

The application of XRD on final quality of stromal collagen tissues analyzed, show differential results according tissue type and / or method of preservation applied. Indeed, relative to the observed results in cryopreservation of arterial vessels we see that regardless of the vessel –carotid or descending thoracic aorta- and the condition of FVA or CVS, a common diffractive peak at d spacing 2.86 Å is seen. The same phenomenon is shown for a second peak at d spacing 2.15 Å. These results show that vascular cryopreservation -defrost procedures did not alter the sequential structure of vascular fresh collagen. In reference to the results of amniotic membrane processing under the same preservation procedures, we see that for both varieties, fresh and cryopreserved, the maximum diffractive profiles remain unchanged: FAS, with d spacing 3.262 Å (28.2 ° in 2 θ) and equal values are checked for CAS. Other 2nd peak at d – spacing 2.359 (40.8º in 2 θ) is verified for both study categories. This confirms that the collagen is resistant to the cryopreservation defrost technique in regard to its sequential molecular structure independently of type of tissue.

Contrary, when both amniotic membrane categories study values are observed we found that FAS variant shows a peak for d spacing of 3.241 Å. (28.4 ° in 2θ), while GAS is expressed in the maximum deflection for spacing d 3.284 (28 ° in 2θ). This lag is not verified for the 2nd peak in both categories that match at d – spacing = 2.359 (40.8º in 2 θ). (See figure 7). These findings showed significant data in the sense that the chemical preservation of amniotic membrane with glycerol, modifies sequencing molecular design of collagen, while this variable is not changed under the cryopreserved defrosted condition.

These would be in according with aforementioned work from (Frushour & Koenig 1975).

Additionally, by analyzing the profiles of ordering by OPC values -in relative terms- we see that both, the cryopreservation defrosted and glicerolización procedures, down modify

profiles designs, defined as "molecular crystals". (FAS vs GAS, OPC value = 14.76; FAS vs. CAS, OPC value = 42.02 respectively).

The main conclusion from these data is: amnion chemical glycerolized procedures, change sequencing molecular design, while physical cryopreserved method does not. But, physical cryopreservation method, and chemical glycerolized, modifies OPC values related to both tissue categories: cryopreserved amnion and vascular tissue.

It must be noted the observed differences in the profiles of diffractive curves between fresh and cryopreserved arterial vessels. Indeed, there are a disparity between those varieties, which were not verified by the corresponding samples fresh and cryopreserved amniotic membrane, that maintain substantially similar profiles. This aspect is independent of the OPC values for both tissues and categories of each study. Our hypothesis is that these differences are related to the anatomical and functional collagen distribution in different tissues. Indeed, the hierarchical order of collagen mesh reach a final design bundles arranged following the lines of force according to bio mechanical requirements.

In this sense, arterial wall of large conduit vessels such as aorta and carotid, are under pulsate hemodynamic regimens alternating expansion and elastic contraction states. Under these conditions, the main loads acting on the vessel wall are pressure and blood flow. The blood pressure acts directly on the inner wall of the vessel in normal direction, and flow proactively work generating a pressure proportional to the square of blood velocity, Fung YC (1997). There are therefore two preferred directions in the distribution of the charges: one circumferential and other longitudinal. This results in a complex morphological organization of the cellular components of the middle layer, composed of smooth muscle cells and collagen mesh ECM, whose design will trace circumferential and longitudinal lines, giving to vascular tissue an anisotropic mechanical behavior condition. (Rodriguez, 2007). The primary ice spontaneous nucleation happen at random in many sites of ECM, Muldrew. 1999). The crystallization growth front, follows the preferred direction lines according to design collagen mesh. According with OPC values, the new organizational picture of the cryopreserved defrosted vascular tissues would be the result of a complex sequence of physic chemical events through preservation procedures, applying changing a complex vascular structural tissue.

This is not the organizational situation of amnion collagen ECM. Amnion membrane has a laminar design, and is not subject to biomechanical pulsate regimen. Its function as an external fetal covering meets fundamentally amniotic liquid metabolic regulations, more than biomechanical functions. In spite of, both kind of tissue studied, (vascular, and amnion membrane) have basically the same fibril collagen composition, namely: Collagen I, III, and V. Additionally, Colagen IV in structural Basements Membranes.

Then, our hypothesis is that the morphologic compositions, and the architectural organizations, according to specific functional requirements to each tissue, define the proper molecular assembly, and therefore its own diffractografic profiles.

8.2 Raman scattering

Our preliminary results obtained on amnion membrane (FAM vs CAS) show punctual differences between both categories in three ranges of Raman Sepectra: 1260, 1442, and 1667 cm^{-1} band (See figure 11) where is observed an increased Intensity (AU) values in FAS

related to CAS. According to references Frank, C. et al (1995), these Raman Spectra range areas aforementioned belong to fibril collagen I, III and V from human placenta. The defined corresponding chemical residues assignments, (Frushour & Koenig 1975). are respectively: Amide III; CH3, CH2 (deform); and Amide I for each range Raman Spectra recorded.

These findings support our work hypothesis about the potential power of cryopreservation procedure to change collagen structure at molecular level.

9. Conclusions

The aim of our study was the analysis of tissues produced in TB for therapeutic purposes, by known techniques, XRD and RS, able to approach the study of structures at the molecular level, mainly in reference to collagen, the fundamental component of ECM,. Taking account the hypothesis that preservation techniques introduce changes in the matrix elements of the tissues, we subjected amniotic membrane and arterial vascular samples under two types of procedures: a) cryopreservation-defrost as physical process, and glycerolization - deglycerolization as chemical process. Regarding to the results of our tests, we accept our hypothesis and concluded that while cryopreservation modifies the structural arrangement of collagen at the level of ECM, glycerolization changes molecular d spacing of biological polymers, besides the aforementioned order. However, the changes between the processed vascular tissues and amniotic membrane are different, because while the vascular cryopreservation increases the molecular order of the crystalline structure measured by OPC value, the amniotic membrane glycerolization and cryopreservation decrease the referred molecular order. These differences are interpreted as the result of complex physicochemical phenomena that occur during preservation procedures on molecular structures and its designs. These phenomena promote variations in the tissue molecular complexity and order distribution. Preliminary data from the Raman tests corroborate the hypothesis of specific modifications in the molecular structures. The consequences of these findings on the allograft biological behavior applied to clinical purposes is a challenge to research and development. Both types of tissues studied are widely applied in the world with beneficial results for the restoration of altered structures and functions in the recipients. But the gold standard allograft is not yet produced, so it is necessary to obtain the allograft that better reproduce the structural and functional conformation of the patient implanted. This objective will be achieved through the best inter relation between recipient structures and the preserved tissues applied, at molecular level to obtain the better possible allograft behavior and patency. Mainly, taking account the advanced development of applied bio engineering, and the design of complex products (composites) that combine different types of cells and artificial, biological, or modified scaffolds.

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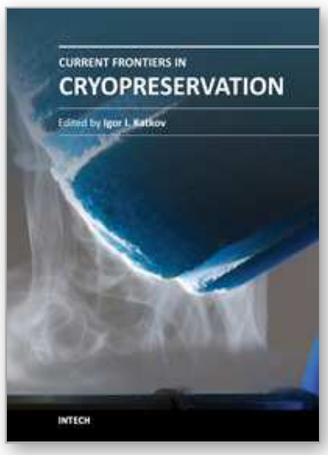

Current Frontiers in Cryopreservation

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Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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