BIOMECHANICAL ASSAYS AMNIOTIC MEMBRANE PRESERVED IN GLYCEROL CORRELATING WITH OPTICAL COHERENCE TOMOGRAPHY (OCT) AND THERMAL GRAVIMETRIC ANALYSIS (TG)

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ABSTRACT

Amnion or amniotic membranes (AM) are interchangeable terms used in the literature being internal part of the fetal membranes, non-vascular and multicellular tissue. The amnion has been widely used as a graft ophthalmic surgical as well as carrier substrate stem cell tissue equivalent for ocular surface reconstruction. The AM reduces scar formation and inflammation on the ocular surface, promotes epithelization also been used as a biological bandage covering the wound or burns, reducing dehydration and allowing regeneration of these areas. The amnion has usually 0.02 to 0.5 mm thick and consists of five subsequent layers: epithelium, basement membrane, compact layer, fibroblast layer and spongy layer. The mechanical strength from the membrane structure as well as the elasticity are factors attractive to the use of amnion as a surgical graft. Higher levels of rigidity and strength may improve the graft resistance necessary to resist the stress induced during growth of the new tissue formed. The amniotic membrane is obtained at elective caesarean section and subsequently, under sterile conditions, sectioned and separated from chorion and placenta, and free blood clots. The serological tests are done at the time of collection of tissue and 6 months after delivery to confirm the results. There are different methods for storing MA in tissue banks as fresh, high concentrations of glycerol, among others. The use of fresh membrane has some limitations due to the need to rapid use and high risk of contamination, however the amniotic membrane in glycerol has antiviral and antibacterial property which is dependent on the concentration, time and temperature. The AM used in transplants must be sterile to prevent the transmission of any disease. Although sterilization by radiation is an effective procedure, it can interfere on the membrane structure. Thus, verification of potential changes caused by ionizing radiation in amnion was made using the tensile test by calculating the Young's modulus, the OCT technique, to generate high-resolution images in real time being a non-destructive technique, thermogravimetry (TG) assessing the amount of water and rate of water output membranes after treatment with ionizing radiation, relating the possible changes with non-irradiated tissue.
However the results of the tensile test had the same behavior compared to the values of total attenuation coefficient by OCT, in addition the dehydration rate analyzed by TG had no statistically significant variation to some radiation doses.

1. INTRODUCTION

The Optical Coherence Tomography (OCT) is a diagnostic technology analysis for non-invasive imaging at microscopic scales. This technique is able to generate images of cross sections of biological systems without the need of any prior preparation of the structures to be studied [1].
The ability of OCT technique is a spatial resolution of 10 μm or less, a resolution which is about 10 times larger than the conventional ultrasonic. Furthermore, unlike conventional ultrasound, this technique does not require direct contact with the tissue being studied. However, because of strong light scattering suffer in most tissues, the depth of imaging is limited to a few millimeters [1, 2].

The OCT system using optical fiber and a compact source of light. The light from the source is split by an optical splitter via which acts as an interferometer. A fiber directs the light to the tissue to be studied and the other directs light to a mirror reference, moving the reference position where the mirror is precisely controlled by an electronic system and a computer. Both the light reflected by the tissue as light reflected from the mirror are recombined and the interference between the two signals occurs only when their lengths correspond to the coherence length of the light. This allows both an accurate determination of the distance into the tissue the light was reflected as its magnitude. The axial resolution of the OCT images generated is determined by the coherence length of the light that was used to obtain the images.

The amniotic membrane is the innermost part of the fetal membranes, consisting of five layers: epithelium basal layer, compact layer, fibroblast layer and spongy layer. The membrane is widely used in transplantation to be a tissue that combines anti-inflammatory and antifibrotic antimicrobial, and limited ability to provoke immunological reactions leading to rejection of the transplanted tissue [3,4,5,6,7,8,9].

After his capture, the elective Caesarean section, processing should be MA in tissue banks and stored in different conditions, such as fresh, high concentrations of glycerol, among others [10]. The use of fresh membrane has some limitations such as the need for rapid deployment and inability to obtain complete safety in the face of certain infections. The other types of processing require the preservation of the same, also allowing sterilization complementary, providing greater safety to tissue [4,11,12,13,14]. The radiosterilization is an alternative to ensure the quality of the tissues being used doses of ionizing radiation sufficient to achieve Sterility Assurance Level (SAL) desired [10, 15,16,17,18].

The use of the technique of thermogravimetry (TG) becomes important to understand the influence of the ionizing radiation sterilization method of the preservation of the tissues. A preservation with glycerol causes the movement of molecules between the collagen network of the tissue and this changes caused by ionizing radiation could affect the flow of that molecules, making it necessary to know if the structural modifications affect the process of water diffusion in the tissue. In this case, the inverse technique that is, checking the release rate of water loss by TG measured by the mass percentage, it can be inferred if there was a change in tertiary structure of collagen, releasing water more easily intrinsic [4, 18]. The thermogravimetric analysis is allowing the continuous recording of mass change as a function of temperature and/or time in a controlled atmosphere. This technique makes it possible to determine the changes that warming could cause mass substances (loss or gain), thus establishing a temperature range in which they acquire fixed chemical composition (mass defined and constant), the temperature at which begin to decompose and verify the progress of dehydration reactions, oxidation, combustion, decomposition, among others. Three modes of thermogravimetry are commonly used: isothermal thermogravimetry, thermogravimetry quasi-isothermal and dynamic thermogravimetry or conventional [19].

One of the mechanical tension tests most common deformation is conducted under tension, can be used to determine various mechanical properties of various materials. In this type of assay, a sample is deformed by a tensile load that is increased gradually and is uniaxially applied along a body proof. The tensile testing machines are designed to stretch the sample at a constant rate, continuously and simultaneously measuring the instantaneous load being applied (with load cell) and the resulting stretching (using an extensometer). Typically, a stress-strain test takes several minutes to carry out and is destructive, the sample being tested is deformed permanently, and often fractured [20].

The materials in general can be classified according to the degree of deformation. Elastic materials regain their original size or shape after deformation without any energy expenditure, since the purely plastic material yield continuously under the action of a force and do not return to the original state spontaneously after removal of the latter, requiring additional strength to return to its initial state. The combination of these two properties is visco-elastic, which has the characteristic after a deformation return to the original state by the elastic component, however, recovery follows that a different curve, but linear due to consumption of the energy applied [21].
The degree to which a structure deforms or flows is related to the magnitude of an applied tension. The stress and strain are proportional to each other by the following correlation:

$$\sigma = E\,\varepsilon \qquad (1)$$

This is known as *Law of Hooke* and unproportionality constant "E" (MPa) is the modulus of elasticity or *Young´s* modulus [20]. The modulus of elasticity (E) is the relation between tension and elongation of the tissue. Thus, the more resistant the tissue to stretching, the greater the value of the modulus of elasticity is calculated as follows:

$$E = \Delta\sigma/\Delta\varepsilon \text{ ou } E = \sigma_2 - \sigma_1/\varepsilon_2 - \varepsilon_1 \qquad (2)$$

Therefore, this study evaluated the use of OCT techniques, TG and tensile test to evaluate the biomechanical changes caused by radiosterilization amniotic membrane preserved in glycerol using two types of sources of ionizing radiation (gamma rays from the cobalt-60 irradiator and electron beam) with different dose rates.

2. MATERIAL AND METHODS

This work was approved by the Ethics Committee of the Faculdade de Saúde Pública of the Universidade de São Paulo. The capture of amniotic membranes was performed after clinical history and serological screening of donors, the Tissue Bank of the Central Institute of the Clinical Hospital of São Paulo (SP-ICHC). The amnion from elective cesarean section, which could not be used for transplantation, were provided by the Tissue Bank of the ICHC-SP, after undertaking formal request through the "Term of Free and Informed Giving" The samples were processed and stored in glycerol concentration greater than 85% for at least 30 days in the Tissue Bank. Membranes were extended on sterile filter paper, with the epithelial side up (smooth and brighter), under aseptic conditions in a laminar flow cabinet. After extended in support of filter paper, the tracks were packed in sterile transparent, sealed and stored in a refrigerator at a temperature 2-8° C until the time of irradiation. Each of the five membranes were divided into non-irradiated sample (control) and irradiated with cobalt-60 source (gamma) and electron beam (E.B.) at doses of 10, 15, 25 and 35 kGy.

2.1. Optical Coherence Tomography (OCT)

The measurement carried out with a light beam incident on a single point in the tissue produces a variation measure of the reflection along the optical axis of the beam. An image of the cross section of a sample produced similarly to the radar: a light beam is launched into the tissue and reflection profiles of different transverse positions are recorded in the computer. The result is a two-dimensional representation of the cross section of the tissue. This representation may then be displayed in a gray scale, or even a false colors (standard RGB), which different colors indicate the degree of scattering of the photons as follows: the white color scale indicates an overflow, or represents a scattering above the upper threshold, the color black represents regions where there is no signal (lower threshold) and the other colors follow the standard RGB (red, Green, Blue), where red represents higher signal, followed by the colors orange, yellow blue, green and violet [2].

The sample rate of 2 x 2 cm was used to perform reading by OCT. The amnion analyzed, removed from the surface of the filter paper was stretched out on a glass slide with a hollow stainless steel support enabling an interface air / air so that the membrane would not be adhered to the glass slide. Were three types of image with different scales (gray colors standard RGB and gray inverted) selecting the best display for data analysis fig. 01.

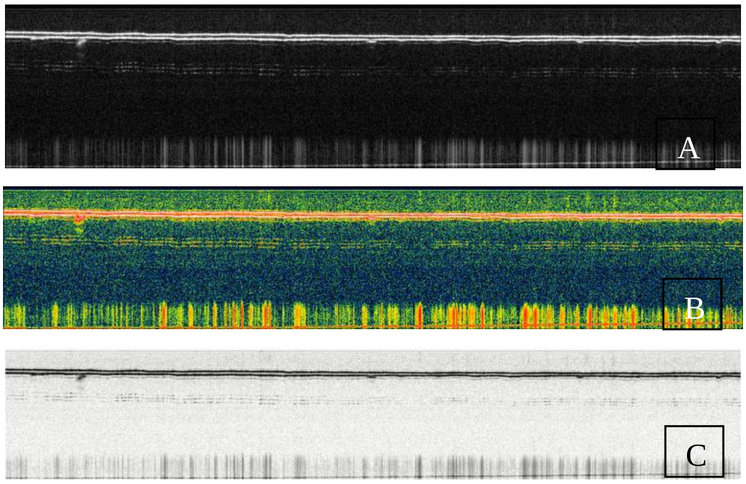

Figure 01: Images of the amnion in glycerol generated by OCT and "A" gray scale "B" scale color "false" (standard RGB) that indicate the scattering of photons and "C" scale inverted gray.

After viewing the images generated was possible to choose the image "A", figure 01A, as standard in the analysis by OCT. The thickness of the amniotic membrane displaying the images generated by the OCT technique could be measured by using Image J software ®. Twenty measurements were performed in triplicate for each image for each irradiation dose in the two sources of ionizing radiation.

Calculation of total optical attenuation coefficient was performed by means of software which allowed the measurement of several areas of the image, generating coefficient doubled because the image has been generated with the way the light path and its return at the time of image registration.

2.1.1. Thermogravimetry (TG)

In this stage, we used the simultaneous thermal analyzer TG / DTA, model Q600, TA Instruments brand. The Q600 analyzer allows for simultaneous data (TG) and differential thermal analysis and differential scanning calorimetry (DTA / DSC), comparing the thermal behavior of the sample against a reference standard thermally inert in the temperature range studied. Alternatively, the device can measure the change in mass of two samples simultaneously, at the expense of data DTA / DSC, behaving like two thermobalances. Thus, samples of 1 x 1 cm previously rehydrated in saline solution for 30 minutes [4], were placed in the balances of equipment for simultaneous evaluation of mass variation. The samples (two by two) were subjected to heating at a ratio of linear 10 ºC min^{-1} to a temperature of 150 ºC, followed by an isothermal stage 10 minutes. Were analyzed: the amount and rate of water output from irradiated and non-irradiated membranes by means of linear regression curve generated by the TA Universal Analysis software, the values of the percent of mass x time.

2.1.2. Tesile Test

Each sample fragment (3,5 x 1 cm) cut in the shape of dumbbell with extensometer 0,5 cm wide and 1,0 cm long fig. 02 has been fixed in the holders and subjected to tensile using the texturometer (Texture Analyzer model TAXT.plus, Stable Micro Systems) with removal speed of 10 mm / minute until failure of the material [22, 23, 24].

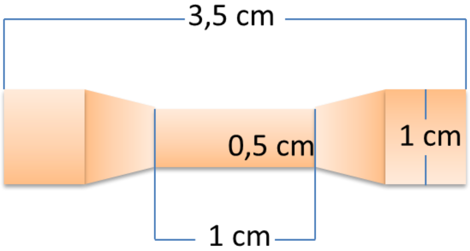

Figure 02: Test body of AM for the tensile test.

After preparation of the samples mentioned above fig. 03A, these were hydrated in saline for 30 minutes fig. 03B and extended in the claws of equipment (texturometer) to perform the biomechanical analysis of amniotic membranes fig. 03C and D.

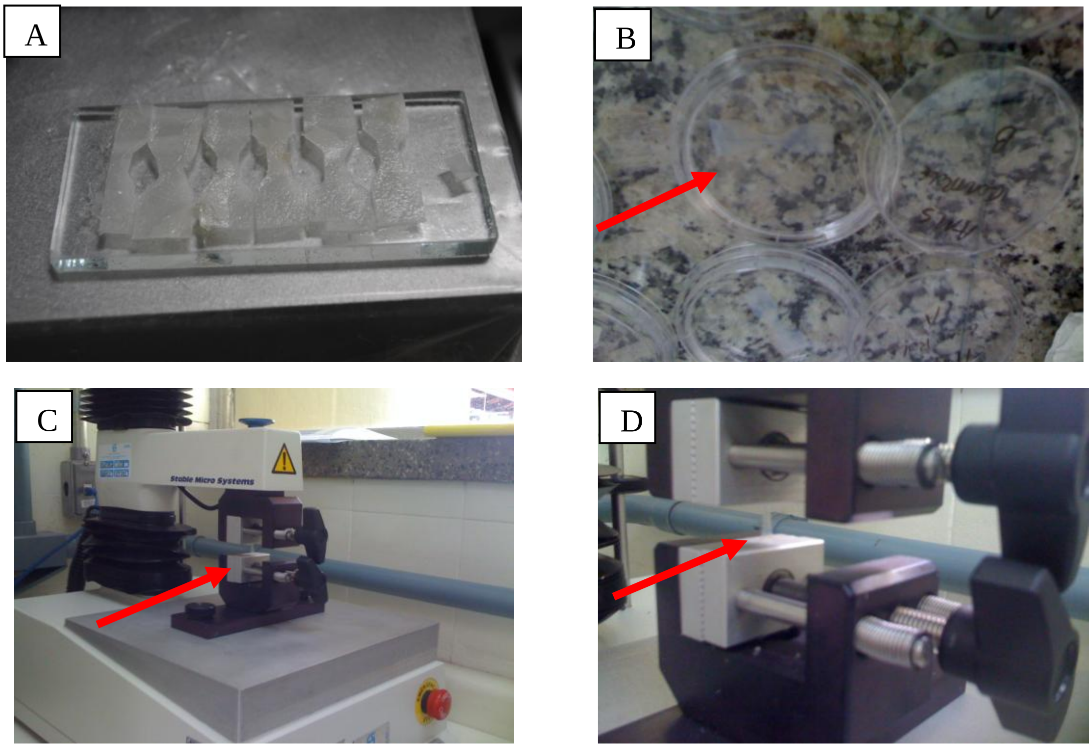

Figure 03: Scheme of analysis of the test bodies made in the form of dumbbells for analysis in texturometer. Image A shows the arrangement of the specimens in the form of dumbbells on filter paper. Image B shows the rehydration step without cutting the filter paper. Images C and D show the fixation of specimens in texturometer for biomechanical analysis.

This test was performed in triplicate for each radiation doses, and effected the calculation of modulus of elasticity (E) the second equation 1 and 2 with the voltage (stress) in MPa and deformation (strain or distance traveled) in mm from the graph generated by the program Advantage v 5.4.0.

3. RESULTS

OCT

The examination of possible changes caused by ionizing radiation amnion was also made using the OCT technique to generate high resolution images in real time, as a non-destructive technique, without prior preparation of the samples. The following images are of amniotic membranes in glycerol or rehydrated, irradiated by two radiation sources in their respective doses.

Although only by viewing the images produced by OCT not be noticeable any changes, one has the impression of increased thickness on membranes irradiated after rehydration. Through the analysis of the images by software Image J ®, were able to measure the thickness variations observed in control samples irradiated in gamma irradiator rays and electron beam. Figure 04 shows the thickness variation as a function of the dose found in the analysis of glycerolated membrane radiated by both sources and after rehydration.

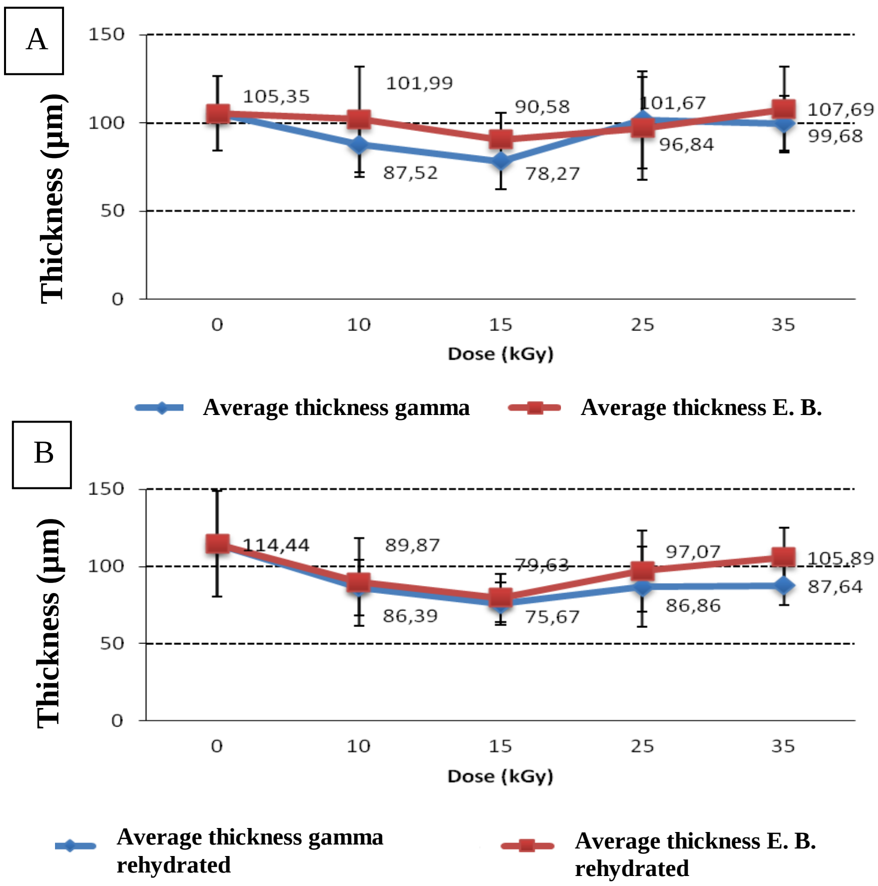

Figure 04: Comparison of the average thickness of amnion glycerol irradiated by gamma rays (blue) and Electron Beam (red): (A) preserved in glycerol and (B) after rehydration.

For a more precise analysis of the results of OCT amnion, the computer program was used for analysis of the total optical attenuation coefficient, developed and updated by Dr. Anderson Zanardi Freitas (2013). The Calculation of total optical attenuation coefficient of tissue is of great value to quantify the differences, both the effect of ionizing radiation on tissues, as to the effects of different forms of preservation fig.05.

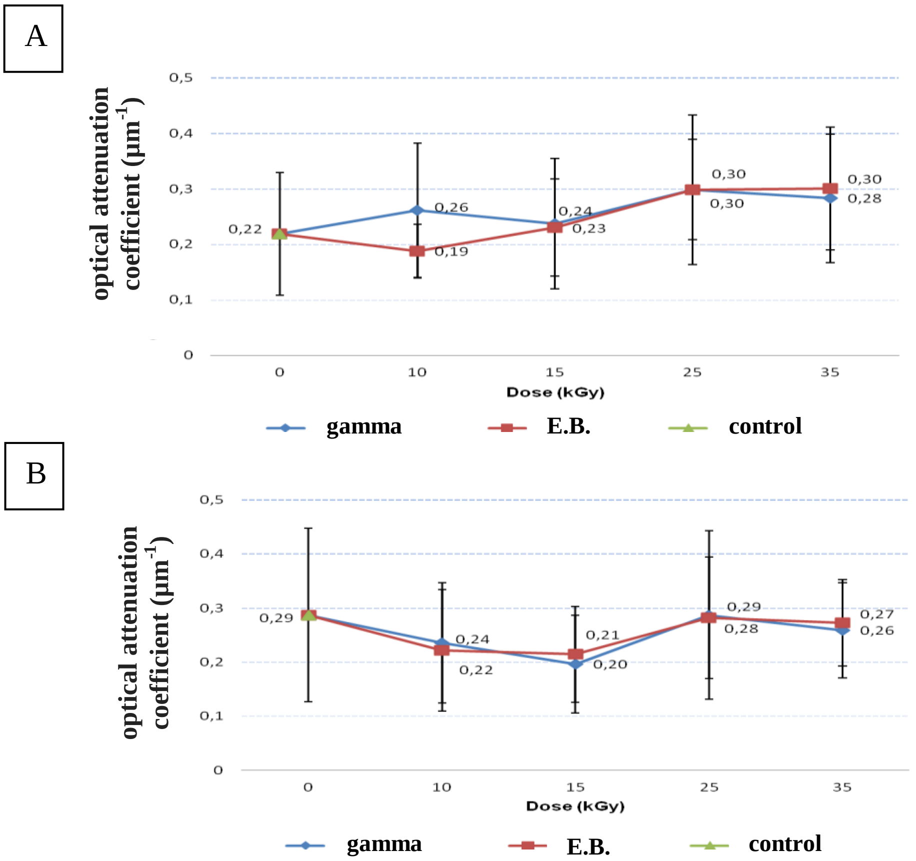

Figure 05: The graph (A) shows the trend of the variation of total optical attenuation coefficient as a function of the dose of ionizing radiation (Cobalt-60 and Electron Beam) of the samples preserved in glycerol. The graph (B) shows the trend of variations after rehydration.

Glycerolated membranes can be observed a trend of increased attenuation coefficient is directly proportional to the dose, unlike what occurred after rehydration of the same, where there was a decrease of these values for the doses of 10 and 15 kGy, in both sources, and at higher doses (25 and 35 kGy) these values tended to approach the non-irradiated control. Statistical analysis showed no significant variation in the cases.

Thermogravimetry

Done calculations of mean linear regression, it was possible to construct the graph of changes in the rate of dehydration fig. 06 in relation to the dose of ionizing radiation used in both radiation sources. The same was done with the mean values of total dehydration membranes analyzed fig. 07, representing the variation of the total moisture of the membranes according to the dose of gamma radiation rays and electron beam, as compared to the non irradiated control.

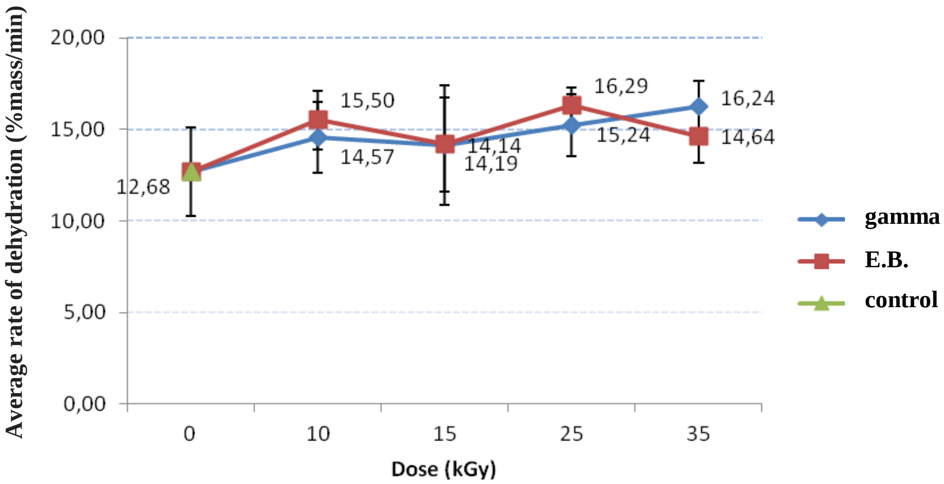

Figure 06: Graphic dehydration rate variation as a function of the dose of ionizing radiation.

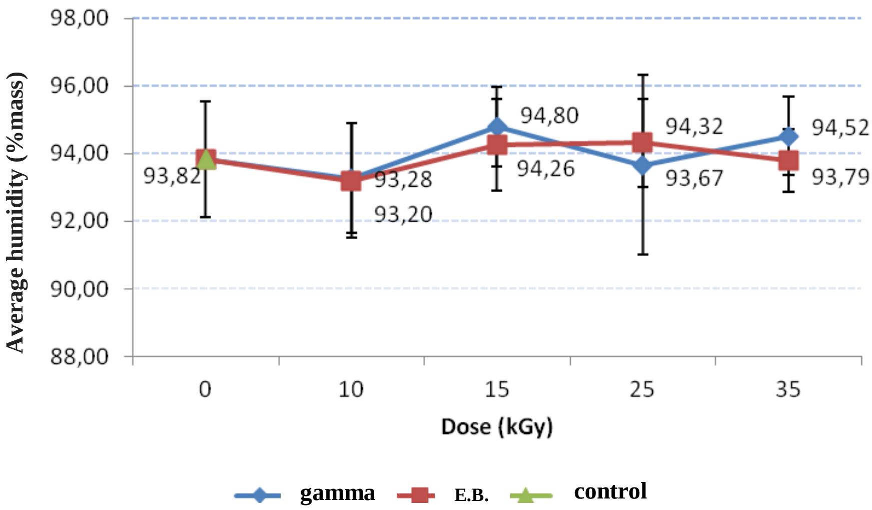

Figura 07: Graphic total humidity as a function of the dose of ionizing radiation of the two radiation source (gamma rays and electron beam).

It was observed that both the rate and total dehydration fig.06 fig.07 membranes showed no statistically significant differences ($p < 0.05$), depending on the radiation dose.

Tensile test

The realization of the tensile test possible to measure the variation in biomechanical function of the dose of ionizing radiation in gamma rays and electron beam. Was calculated average elasticity modulus by dividing the tension by deformation of the tissue.

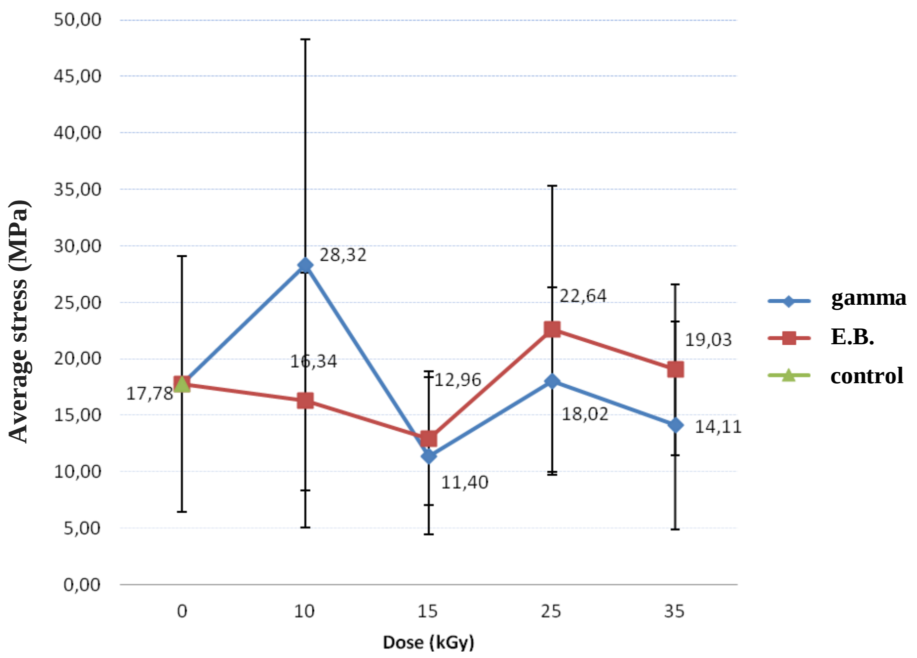

Figure 08: Ratio between the average stress rupture and the dose of ionizing radiation to the amnion samples irradiated in gamma rays, electron beam and control.

Using data from the averages of the results, a graph was plotted of modulus as a function of radiation dose of Cobalt-60 source and E.B. fig.08. It was not possible detect statistically significant variations ($p < 0.05$) in these test conditions.

4. DISCUSSION

Through the analysis of images generated in OCT technique which enabled great progress in studies of ultrastructure of bone and cartilage [18], but it was not possible to differ the layers in the structure of glycerol-preserved amnion, however it can view their thickness full. Images were also made of the same membrane after hydration in 0.9% saline solution for 30 minutes. Initially, analyzes were performed only with the use of the images generated by OCT and analyzed in Image J ® computer program that is used to measure the different thicknesses of the amnion according to radiation doses. In relation the average thickness of all membranes in the dose and type of radiation source, it was observed that the variations in thickness of the samples rehydrated much as glycerol, were not statistically significant figure 04.

Subsequently, the images generated by OCT were analyzed by calculating the total attenuation coefficient however, despite the results present a trend of variation with increasing dose of radiation (gamma and E.B.) this change was not statistically significant fig.05. This is probably due to the fact that the membranes have a small thickness, near the lower limit of detection of the equipment [2].

Thus, the thermogravimetric test was conducted to evaluate this trend found in the analysis of the variation in OCT rehydration capacity of the membrane after irradiation. Using the mean values of the rate of dehydration and total loss of volatiles (water) were prepared with variations graphs of doses and types of source fig.06 and 07. Likewise the OCT data, the results showed no statistically significant differences in relation to dose or source applied.

This fact is explained in the study of MARTINHO (2012), which found that the means of preservation in glycerol cancel the differences caused by doses of ionizing radiation to thermogravimetric curves because the dehydrated the tissue, its characteristics are homogenized.

In order to identify structural changes in the membrane, was initiated biomechanical analysis (tensile test), where the tests were performed simulating the clinical application of amnion graft, in which this would be extended to cover the surface of a wound. After rehydrating the membrane by analyzing the values obtained texturometer stress and strain for calculation of modulus, thus, it was possible to construct the graph, fig. 08, the elastic modulus versus dose variation, to compare the results of the samples irradiated in gamma rays and electron beam relative to non-irradiated control. However, as the OCT and TGA analysis, even analyzing the membranes five for each dose in triplicate (n = 15) was not able to detect statistically significant variations in these test conditions.

In the study by Nakamura (2004), biomechanical test was performed in two different ways: first testing and second tensile way by breaking at the point of suture. However, with a six samples (n= 6), the results were not statistically significant between samples gamma irradiated at 25 kGy and not irradiated.

Chua & Oyen (2009), report data are inconclusive about the elastic properties of membrane in relation to the modulus of elasticity. In soft tissue, depending on the tensile level and the rate of deformation, in general, additional data (n> 15) to construct a graph non-linear and more accurate results and detailed viscoelastic nature of the membranes. Similar to observations of Chua & Oyen (2009), in our study, even using n= 15 (triplicate samples of the five membranes), the analysis of tensile has not presented a statistically significant difference between the samples tested, only being possible this viscoelastic behavior in the amniotic membranes irradiated.

5. CONCLUSIONS

Using the techniques of OCT, TG and tensile testing was not possible to evaluate the findings in quantitative analysis, under our experimental conditions due to the deviations obtained for the five membranes tested. Thus, should be increased sample number and other techniques such as solid colorimetry and optical microscopy should be evaluated by providing a better assessment of change in amniotic membrane.

ACKNOWLEDGMENTS

We would acknowledge the International Atomic Energy Agency (IAEA) and the Comissão Nacional de Energia Nuclear (CNEN), for supporting this work.

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