MORPHOLOGICAL ANALYSIS OF AMNION STORED IN GLYCEROL STERILIZED WITH DIFFERENT DOSES OF IONIZING RADIATION

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ABSTRACT

The amniotic membrane (AM) is the innermost layer of the fetal membranes (placenta), widely used in transplantation being a tissue that combines anti-inflammatory, antimicrobial and antifibrotic, and limited immunogenecity. The tissue can be used a bandage biological for treatment of burns and skin wounds, chronic ulcers, reconstructions from different body areas, including ophthalmic repairs. In the last decades the amniotic membrane has been used widely also as a carrier substrate to transfer tissues cultured "in vitro". The use of fresh membrane has some limitations, the main ones are being necessary your quick use and the inability to obtain full security for certain infections. Other types of preservation require a processing thereof. The radiosterilization is an alternative for ensuring quality and safety of tissues used in transplants, and other clinical applications in order to minimize the risk of contamination of the receptor tissue. Therefore, the objective of this study was to test various doses of radiation using two sources of ionizing radiation: the cobalt-60 irradiator Gamma and Electron Beam Accelerator (E.B.). A tissue analysis was done by visual and tactile qualitative analysis, semi-quantitative (solid colorimetry) and light microscopy to observe morphological and physic-chemical changes after the irradiation of AM preserved in glycerol, comparing the results obtained with the sample not irradiated. It was noted that at higher doses, for both radiation sources, irradiated membranes suffered greater color change, becoming yellowish and thereby reducing their initial malleability.

1. INTRODUCTION

The search for factors that stimulate tissue regeneration favors the emergence of alternative therapies with the goal of replacing conventional transplants and complications in organs and tissues, for simple and secure mechanisms for restoring damaged areas of the body [1,2].
The amniotic membrane (AM) is the innermost part of the fetal membranes composed 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]. According Dua & Azuara-Blanco (1999) [9], this tissue serves as a biological bandage for treatment of burns and skin wounds, chronic ulcers and reconstructions of various areas of the body, including ophthalmic repairs.
After their capture in elective Caesarean section, the processing to be of AM in tissue banks and stored in different conditions as follows: a fresh, high concentrations of glycerol, among other [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 tissue being used doses of ionizing radiation sufficient to achieve the *Sterility Assurance Level* (SAL) desired. Products which are in contact with blood, parenteral solutions, as well as tissue grafts require SAL de 10^{-6} which means one in a million probability of remaining viable microorganism in a unit of product [9,15,16,17,18].

In tissue banks, the selection of the dose of irradiation for sterilization remains a controversial topic. Are selected radiation doses from 15 to 50 kGy for sterilizing the amniotic membrane. According to the literature, the dose most commonly used in the UK, U.S. and many other countries is 25 kGy, and the Scandinavian countries, it is recommended between 35 kGy and 45 kGy [2, 9; 17,19;20].

High dose (50 kGy) may be necessary for the sterilization of tissues which have a viral load level, but these can cause various physical and chemical changes in the tissue. [9] According Paggiaro (2011) [2], doses of 25 kGy alter the basal membrane of MA preserved in glycerol, affecting the growth of new cells autologous on this tissue this change is more evident when the tissue is irradiated with electron beam accelerator compared to irradiation of gamma rays from Cobalt-60 source.

Thus, in this study we evaluated the 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 Giving Free and Informed " 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. Qualitative analysis

The manual and visual qualitative analysis of samples of size 2 x 2 cm (triplicate) was performed in membranes preserved in glycerol and subsequently rehydrated by observing the visual morphological characteristics of the tissue and after treatment with ionizing radiation and possible changes relating to the non-irradiated tissue. The results were analyzed using photographic documentation and comparison frames in the parameters of color and elasticity, among the samples irradiated in the two radiation sources and control.

Table 1 – Model assessment of qualitative analysis for color

0	No significant change compared to the control non-irradiated
1	Little change yellowish
2	Moderate yellowish color change
3	High change yellowish

Table 2- Model assessment of qualitative analysis for Elasticity → Viscoelasticity

0	No significant change compared to the control non-irradiated
1	Little loss of elasticity
2	Moderate loss of elasticity, increased viscoelasticity
3	High loss of elasticity, increased viscoelasticity

2.1.1. Solid Colorimetry

In colorimetry, we analyze the characteristics of the color defined by three attributes: the attribute of brightness and chromaticity of the two attributes (L *, a * and b *). The color changes of the samples of size 2 x 2 cm (triplicates), of the amniotic membranes stored in glycerol and subsequently rehydrated were analyzed using the Gardner Colorimeter spectro-guide sphere, Byk gloss.

2.1.2. Histological staining

The sections amniotic membrane dimension 1 x 1 cm were rehydrated for at least 30 minutes and packed in flat-bottomed container with a solution of 10% buffered formalin for 24 hours, then in 70% alcohol solution. Histologic paraffin embedded in the membranes were cut with a microtome cross-sections of 5 µm in thickness. Staining was performed in the cross-sections by hematoxylin-eosin (HE) on glass slides in order to observe the changes of the layers of the amniotic membranes.

3. RESULTS

Qualitative analysis

Was performed in triplicate for each dose for both radiation sources (gamma and electron beam), the membranes preserved in glycerol (fig.01) and subsequently rehydrated. The tests were conducted concurrently with the solid colorimetric test, which was required to handle the colorimetric analysis of samples. The degree of variation in color and elasticity parameters were assessed according to the scale of the previously mentioned degree of change (Table 1 and 2).

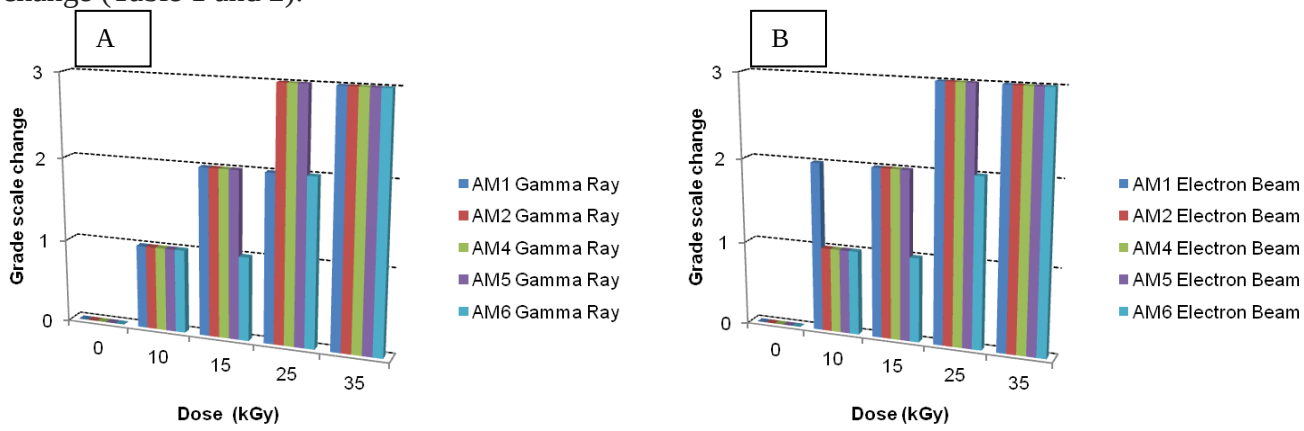

Figure 01: Comparison of the changes in elasticity of the amnion found in five different membranes glycerolated and irradiated gamma rays (A) and E.B. (B).

The change in staining was observed in all samples, ranging in intensity with increasing dose. Photographically (fig.02), has been difficult to assess changes of the membranes color after irradiation that was seen with the naked eye, both membranes preserved in glycerol as the membranes rehydrated.

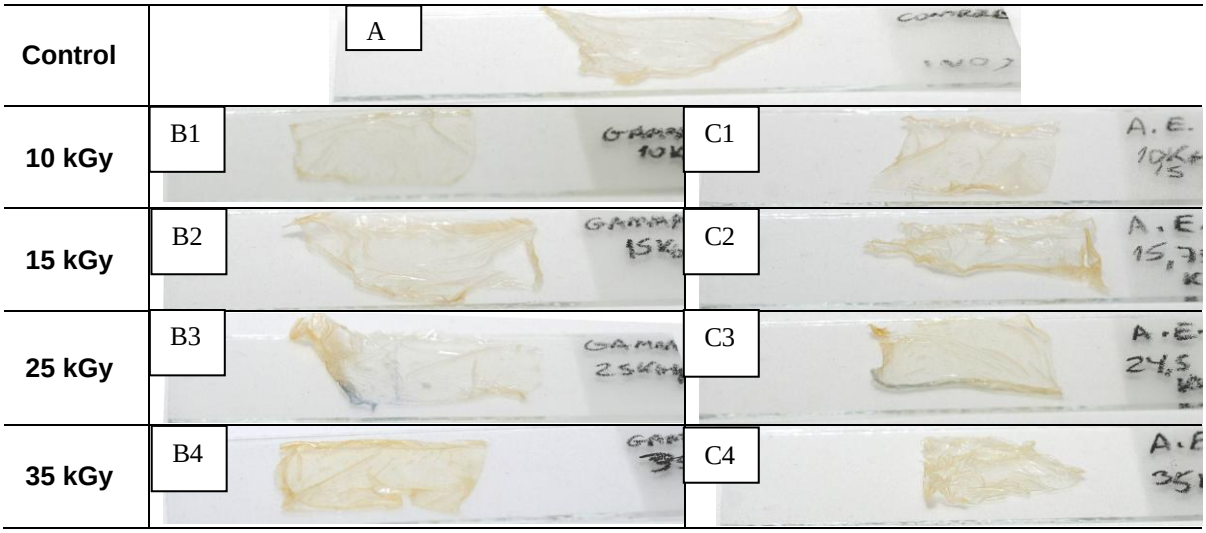

Figure 02: Comparison Photographic color changes of the samples of amniotic membrane in non-irradiated glycerol (A) irradiated with cobalt-60 (B1-B4) and irradiated with E.B. (C1-C4).

In the analysis of the parameter of elasticity were homogeneous among themselves until a dose of 15 kGy (fig.03). Since the same results were obtained after rehydration was not performed graphical representation thereof.

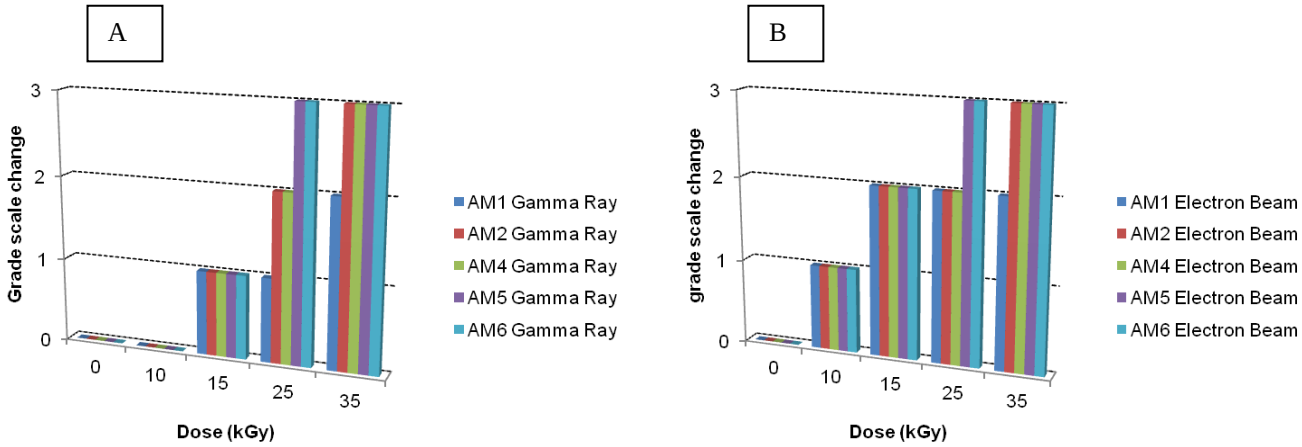

Figure 03: Comparison of the changes in elasticity of the amnion found in five different membranes glycerolated and irradiated gamma rays (A) and E.B. (B).

Solid Colorimetry

With the measurements of the five membranes made by the colorimeter analyzed, the mean results were represented in the figure, both the membranes stored in glycerol after rehydration, for each dose of gamma radiation and electron beam applied in addition to the controls non-irradiated. Starting from the control non-irradiated up to a dose of 15 kGy (Gamma and E.B.) the average of the values varies axis "b *" 2.5 to 4.0, and the axis "a *" from -0.45 to -0.52. From 25 kGy (Gamma and E.B.) the change in axis "b *" 5.0 to 6.0, and the axis "a *" from -0.75 to -0.9 (fig.04 A). After rehydration, the same behavior was

observed between the mean values, ranging from "b *" 2.5 to 5.0, and the "*" from -0.5 to -0.75, and up to 15 kGy from 25 kGy, 6.2 to 8.0 (+ b *) and -0.8 to -1.1 (-a *) (fig.04 B).

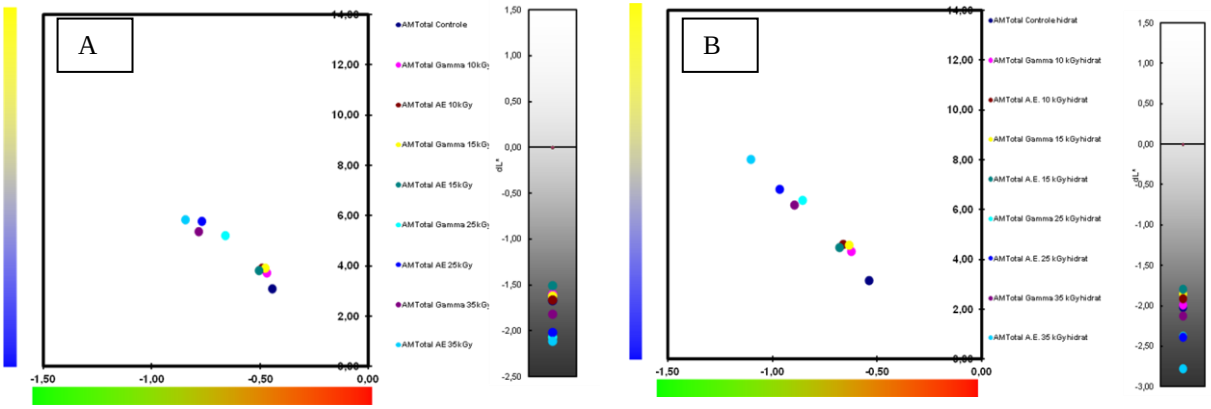

Figure 04: In (A) are the results of the analysis of mean amnion preserved in glycerol (control and irradiated gamma and E.B.), with their respective doses. In (B) the results are the averages of the amnion after rehydration (control and irradiated gamma and E.B.), with their respective doses.

A statistical analysis of variance values colorimetric (dE *) were constructed graphs (fig.05), which propose that the variation in color of amniotic membranes preserved in glycerol had statistically significant from 25 kGy (fig.05 A), while after rehydration thereof, the change in coloration, compared to non-irradiated control was statistically significant at 10 kGy (fig.05B).

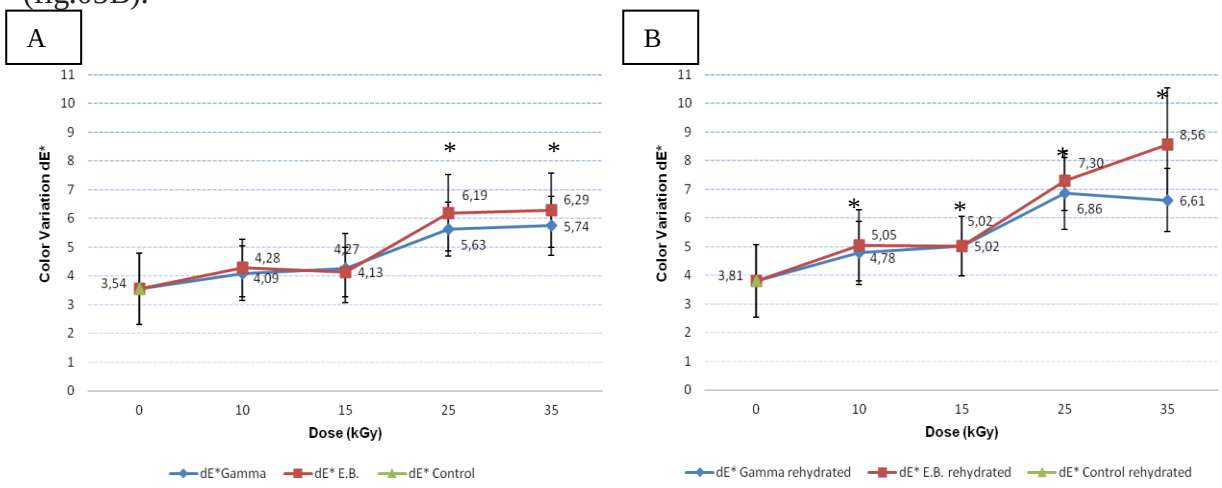

Figure 05: Change in color of the samples preserved in glycerol (A) and the variation of the samples rehydrated (B).The symbol "*" was used indicate significant statistical differences between the control ($p<0.05$).

Histology

After HE staining, the samples were analyzed in an optical microscope at 400x magnification and can observe the constituent layers of amniotic membrane, with the uppermost epithelial layer stained cells and the other in purple stained in pink (fig.06).

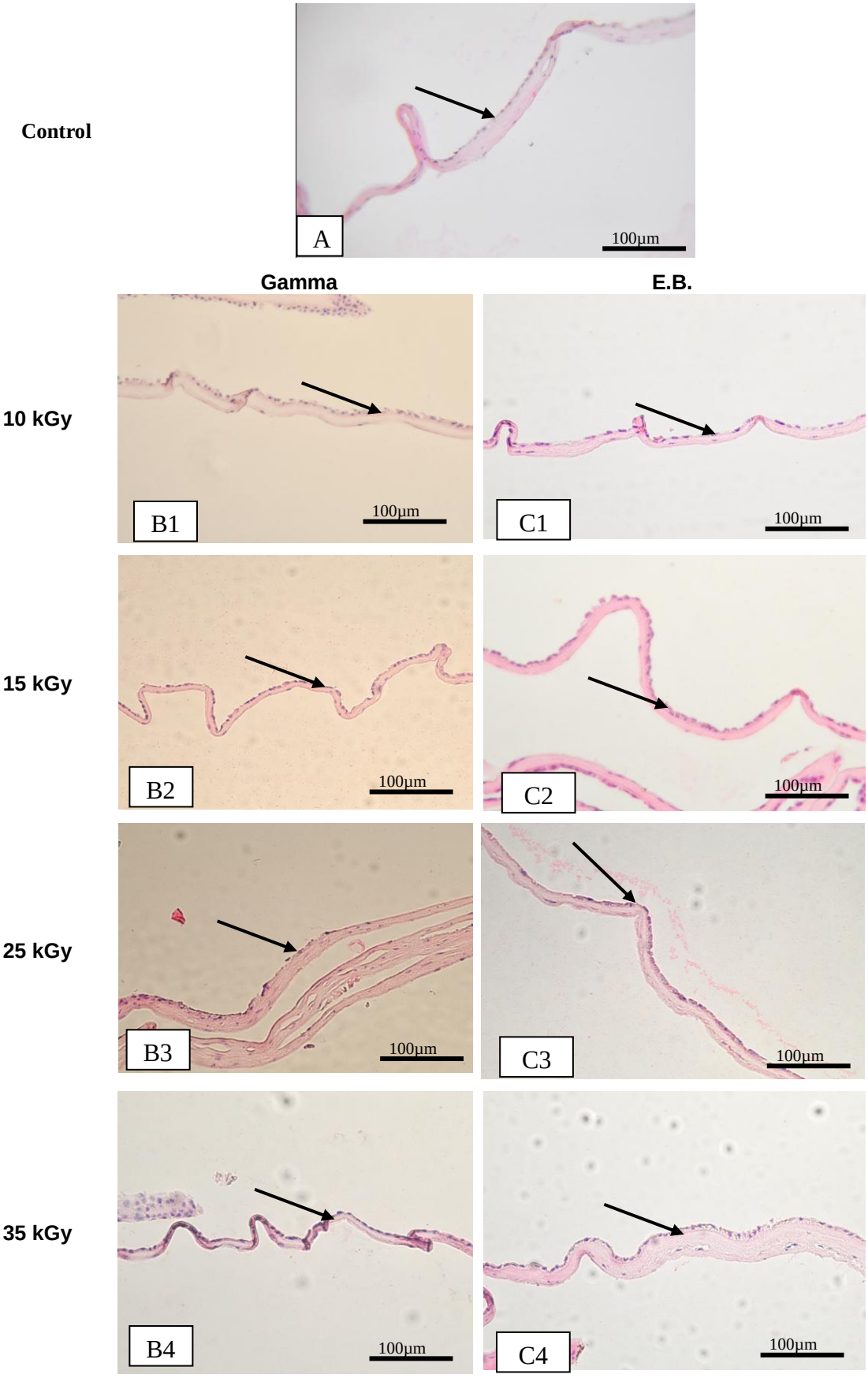

Figure 06: AM1-Photomicrograph of non-irradiated (A); radiated gammal (B1 to B4) and E.B. (C1 to C4), at 400x magnification. (↘) Indicates positioning of the epithelial layer.

In figure 06 A (non-irradiated control), it was possible to observe the epithelial layer with well-defined cells, represented by the arrow in black, as well as their underlying layers. The samples irradiated at 10 kGy with gamma and E.B., figures 06 B1 and C1, respectively, appear to be low cell loss in its epithelial layer and preservation of its underlying layers, as seen in the samples irradiated with 15kGy, figures 06 B2 and C2. The samples irradiated at 25 kGy from appear to be thicker figures 06 B3 and C3, as seen when irradiated with 35 kGy figures 06 B4 and C4.

4. DISCUSSION

The use of amniotic membrane, much as biological bandage, and as a substrate for cell culturing skin or eye, is of paramount importance to prior assessment by the physician transplantador. This first analysis is performed in a qualitative way, through manipulation of the material, giving an idea if it is in working condition. Nakamura (2004) [21] in their study of lyophilized membranes, noted that after the process of storage and sterilization, the membranes appear to be very light, thin, easy to handle and suturable, transmitting soft flexibility after rehydration, similar to membrane cryopreserved.

In the present study, it was observed that the membranes preserved in glycerol, when irradiated with doses up to 15 kGy in both radiation sources (Gamacell and E.B.), had little to moderate color change (tab.01); already at higher doses from 25 kGy, the five membranes were analyzed for each group of moderate to high color change, showing more yellowish aspect (fig.01).

Change in color was observed in all samples, ranging in intensity with increasing dose. Photographically (fig.02), it was difficult to assess changes in membranes color after irradiation that was seen with the naked eye, in both membranes preserved in glycerol as the membranes rehydrated. The evaluation of this change is not perceived visually evident when photographed. Although the qualitative analysis of the parameter of elasticity (tab.02) and in order to evaluate macroscopic changes regarding the clinical application of amniotic membrane as well as qualitative analysis of color were observed tactile variations among groups. Thus, at doses from 15 kGy to moderate little variations are noticeable, both irradiated samples by Gamma such as E.B., with no variations between the membranes of the same group. At doses of 25 and 35 kGy, there are small variations between the membranes, but all changes were observed elasticity with character homogenization result with dose of 35 kGy irradiated samples by both sources (fig.03).

Thus, using the solid colorimetry became possible to accurately analyze the color changes undergone by the material according to the dose applied. Mean results of the samples in glycerol (n=45) at doses of 10 and 15 kGy by both radiation sources was the same both in the variation range of yellow-blue (+ b *) and the scale red-green (-a *) and similar to the average value obtained in the controls (non-irradiated samples). The result of the analysis of samples in glycerol irradiated with 25 kGy and 35 kGy was greater variation in both scales (+ b * and -a *) compared to the results of the colorimetric averages of non-irradiated controls (fig.04 A).

In evaluating the average of the membranes when rehydrated and irradiated with 10-15 kGy, the behavior of color changes were similar to the mean variations of the samples preserved in glycerol irradiated with the same doses of both gamma rays emitted by source cobalt-60 as in electron beam accelerator. Already at doses of 25 and 35 kGy, the change in color of the samples was rehydrated up to changes of the samples in glycerol medium, with higher values

of the yellow-blue scale, with higher results in displacement of axis - a * to green, which results in a predominantly yellow-green coloration (fig.04 B).
As for the luminosity, there was a small shift dose-dependent scale white-black (-L *), observing a greater displacement only in samples irradiated and rehydrated in E.B. with higher doses (35 kGy). In the other groups, the variation in luminosity between the irradiated samples preserved in glycerol and rehydrated remained similarly the same (fig.04 A and B).
Performing statistical calculations for average values of variation in color (a *) compared to control non-irradiated (fig.05), it was observed that the change in color of the samples preserved in glycerol were statistically significant result ($p < 0.05$) dose of 25 kGy from (fig.05A), but after rehydration thereof, it was found that the change was statistically significant ($p < 0.05$) in a dose from 10 kGy (fig.05B), or even the lowest dose radiation employed by both sources (gamma and electron beam) caused changes in color membrane.
VERSEN-HÖEYNCK (2004) [3] mentions that despite the sterilizing radiation (gamma and electron beam), introduced in 1955, is known to be effective in appropriate doses, there is interference on the tissue structure and the biophysical properties. Thus, we sought to elucidate the histology morphologically changes in qualitative analysis, the elasticity parameter by hematoxylin-eosin (HE) [2]. In the upper portion of the membrane, there is the presence of epithelial cells formed by well joined to each other, the basal lamina separating it from the other layers of tissue (collagen), this being fully and continuously as well, the density of the compact layer and fibroblasts can be distinguished by HE staining intensity and the presence of fibroblasts, respectively, and the foamed layer is not clear due to cleaning processes occurring in the handling of the membranes (fig.06 A). In doses of 10 kGy in fig.06 Gamma B1, the histological sections show an early fragmentation and preservation of the epithelium from the underlying layers, apparently unmodified. This was most evident for the samples irradiated with the same dose in E.B..
The samples irradiated in Gamma dose of 15 kGy (fig.06 B2) present fragmentation of the epithelial layer similar to the dose of 10 kGy to the same source, with apparent preservation of the remaining layers of the amnion. But the samples irradiated in fig.06 E.B. C2, with a dose of 15 kGy, besides presenting fragmentation epithelium, become noticeable condensation of the remaining layers of the amnion. In samples subjected to a dose of 25 kGy in fig.06 Gamma B3 epithelial cells are scarce, and the other layers are shown merged together. In samples irradiated with 25 kGy, in E.B. (fig.06 C3), it is observed that the epithelial layer is present with relative cell number, but slightly misshapen, and apparent condensation of the other layers. In the latter case, probably condensation of all layers to prevent fragmentation of the epithelial layer. In the sample irradiated up to a dose of 35 kGy Gamma (fig.06 B4) was observed condensation of the layers, including the presence of epithelium. The same applies to the samples irradiated in E.B. (fig.06 C4), which are more evident in the changes mentioned above.
VERSEN-HÖEYNCK (2004) [3] depicts the change in the amniotic epithelium after treatment with radiation sterilization, the most impressive, the emergence of large intracellular spaces and degeneration of amniotic epithelial cells. This fact was also reported by AB HAMID (2012) [14], at doses of 35 kGy samples preserved in glycerol, as in samples preserved with the technique of air dried, these changes are seen with a dose of 25 kGy.

5. CONCLUSIONS

In the qualitative analysis, it was observed that higher doses (from 25 kGy) to both sources of radiation irradiated membranes had a greater color change, becoming more yellowish and

with a decrease in elasticity, making it more rigid. These changes were generally intensified doses applied to the electron beam.
In colorimetry solid and optical microscopy, it was observed that the color change of the membrane and the degree of condensation of the underlying MA membranes is directly related to the dose of radiation, respectively.

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