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OPTICAL AND HISTOLOGICAL EVALUATION IN HUMAN TENDON TISSUE STERILIZED BY IONIZING RADIATION

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ABSTRACT

Sterilization by irradiation is a technique that is used by tissue banks aiming to eliminate contamination of human allografts, being a safe method, free of residue and used as final sterilization. After the tissue procurement, these undergo a series of processing stages and then are packaged and preserved by freezing. Despite aseptic care of the material those may be subjected to sterilization in the final packing by ionizing radiation, raising the security level of sterility of the tissue. The aim of this study was to evaluate the effects of application of ionizing radiation, produced by ^{60}Co source in human tendons preprocessed (A-alcohol + antibiotic; B- H_2O_2 + ultrasound) obtained through collaboration with tissue banks and preserved by freezing in -80° C, the radiation absorbed doses in processing were 12.5, 15 and 25 kGy, each one with their corresponding non-irradiated control, to examine possible structural or morphological alterations. The irradiated samples and their controls were analyzed by means of optical coherence tomography (OCT) and optical coherence tomography polarization sensitive (PS-OCT), and histological tests had been stained with hematoxylin-eosin (HE). According to the results the tissue processed with alcohol/antibiotic in conjunction with irradiation proved to be the most effective.

1. INTRODUCTION

Sterilization of allografts is required to reduce the risk of transmission of infectious agents. For being a definitive method for elimination of microorganisms and prevent life-threatening infections associated with these [1].

Different techniques of sterilization have been used to prevent infections by allografts, including gamma irradiation [2].

The IAEA standards recognize the fact that many of the irradiation facilities are available to sterilization of tissue processed by tissue banks, using the technique of ionizing radiation, through the application of different doses of radiation.

According to the ISO 11137, when the result of microbial load is equal zero colony-forming units, minimum 15 kGy dose may be applied. The safety levels recommended in ISO 11,137 of 2006 are of 10^{-6} SAL (*Security Assurance Level)*, that is, the probability of finding one part per million of colony forming unit after the sterilization process [3].

The effectiveness of the use of gamma radiation as a sterilization method is due to its great ability to penetrate the matter and its high efficiency to kill microorganisms, in inactivating them and allowing sterilization of materials that were previously in sealed pack, thus preventing subsequent recontamination [2].

With the growing interest in the development of less invasive techniques for tendon and ligament reconstruction surgeries, research on the use of allografts have increased [4]. Tendon grafts can be obtained from multiple organ donors or from stopped hearts. There are significant advantages in the use of grafts obtained through multiple organ donation, such as tissue transplant coordinators can ensure that donors are adequately identified and examined to secure the health condition. In addition, it is essential that the grafts are collected aseptically using standard operating room protocols, in conjunction with aseptic surgical techniques for the procurement, processing and storage [5, 2].

However, with the use of allografts exists the risk of contracting infectious diseases from the donor, but also there is the possibility of microorganisms to be introduced during the procurement, preservation and storage of tissues [6]. With the purpose to eliminate potential contamination, donor screening is essential, musculoskeletal allografts are usually disinfected using antibiotics, irradiation or chemical methods varying according to the protocols of the tissue banks.

As part of processing, in general many banks consider necessary a final sterilization by means of gamma radiation in general from ^{60}Co sources [2].

Therefore, it becomes important to study the possible tissue alteration caused after the procedure of sterilization by irradiation at different doses to determine the appropriate dose for sterilization of this tissue, providing security in sterility and in the structure of the material to be used for transplant.

2. MATERIAL AND METHODS

2.1 Material processing and irradiation

Before the performance of the tests, the study was submitted and approved by the Ethics Committee of Brazil Platform under the number CAAE 44669115.0.0000.0065.

The samples of musculoskeletal tissue were obtained with the collaboration of two institutions: Santa Casa de Misericórdia of São Paulo and Institute of Traumatology and Orthopedics of the Clinicas Hospital of Medical School of São Paulo.

The samples were processed by two methods, A and B, in clean room, whit area class 5 according to ABNT NBR/ISSO 14644-1 as follows: after the procurement of the tissues, a tissue cleaning process was performed, to remove adhered muscle and other soft parts or tissues of the tendon, with the aid of a straight chisel or rugina and scalpel. Being previously cleaned the tendons processed for method A were immersed in a liter of saline solution 0.9% for 15 minutes and soon after in a solution of 70% alcohol for 15 minutes and finally the tendons were immersed in a liter of solution containing antibiotic (500 mg of Vancomycin per liter of saline). Then, it was carried out collecting material for microbial analysis (swab of each tissue) and collecting material for bioburden of the last liquid in contact with the tissue. To finish the process the tissue was placed in a container of sterile plastic, vacuum-sealed and stored in freezer-80 °C.

The processing B: After the procurement of the tissues, these have gone through a process of cleaning, effecting the withdrawal of adhered muscles and other soft tissues of the tendon. Soon after cleaning, the tissue was placed in the ultrasound along with a solution of 3% hydrogen peroxide, for a cycle of 200 to 300 seconds. After processing, the tissue was vacuum-packed in sterilized plastic bags and stored in freezer-80 °C.

The samples were separated according to the received processing, batch, with their respective control. The identification of the samples was made according to the Bank applied method processing and irradiation dose applied being control (non-irradiated); 12.5; 15 and 25 kGy. For irradiation samples were placed in thermic boxes lined with cardboard, separated by dose, being the material settled on the box face and completing the rest of the space with dry ice, to avoid temperature variation. The process of irradiation of samples was performed at the Technology Center of Radiation (CTR) of the Nuclear and Energy Research Institute (IPEN-CNEN SP). Samples of the different groups were gamma irradiated, using ^{60}Co sources of Multipurpose Irradiator of compact type and received effective doses of 12.6, 14.6 and 23.9 kGy, corresponding to nominal doses of 12.5, 15 and 25 kGy, whit a dose rate of 4.2 kGy h^{-1}. Control samples were maintained under the same conditions, however, were not irradiated.

2.2 Optical Coherence Tomography (OCT)

The samples were analyzed in the Center of Lasers and Applications of Nuclear and Energy Research Institute.
The OCT system OCP930SR (Thorlabs Inc. NJ, USA) uses as optical source a Superluminecente LED (SLD) with a spectral width of 100 ± 5 nm and central wavelenght in 930 nm, with power of 2 mW in the sample (from 0.5 mm wide by 0.4 mm length). The images with a lateral

resolution of 6.0 microns and 4.1 longitudinal microns were generated by the displacement of the point of incidence in the sample by mirrors attached to a galvanometer. The light was focused in the sample with a 5 cm lens focal length. The data obtained and stored in microcomputer. Two images were obtained, one of each of the methods to observe the structure of samples.

2.3 Polarization Sensitive Optical Coherence Tomography (PS-OCT)

The OCT has a module of polarization PS-OCT 1300 (Thorlabs Inc. NJ, USA), which is used for optical coherence tomography test is sensitive to polarization, it has a wavelength centered on 1325 nm with 10 mW of power, of which images were obtained at an angle of incidence of light approximately 45° along the sample axis. The images made in PS-OCT, highlighting the birefringent areas can provide quantitative measures of birefringence and penetration depth of light, which are continuous variables measured in levels of reason [7]. The images were obtained and stored in microcomputer. A total of 64 images were obtained of the method A samples, being 32 of non-irradiated tissue images and 32 of the same tissue after irradiation and from method B samples were 72 images, 36 of the non-irradiated tissue and 36 after irradiation.

2.4 Analysis of data obtained by PS-OCT

For the statistical analysis of the difference of distances between the birefringent areas, 40 measurements were carried out in micrometers using the birefringent area with the *ImageJ* software [8] in each of the 136 images obtained. The values of the averages were submitted to analysis of variance (ANOVA) unifactorial. For all variables were calculated measures of descriptive statistics, such as average, median, standard deviation and range standard interquartile (IQN), to verify that there was a statistically significant difference ($p < 0.05$) between the control and the irradiated samples. When there were differences, $p < 0.05$, the Tukey test was applied to the comparison of averages. for the inferential analysis was considered a value $\alpha = 5\%$ for rejection of the hypothesis of invalidity [9]. For the statistical analysis was employed the software *GraphPad Prism* 5.

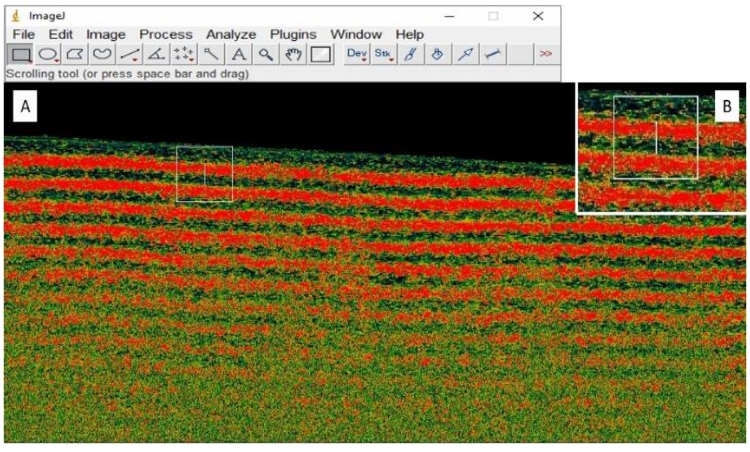

Figure 1: Image A measurement performed using ImageJ software, measuring the distance between the bands of birefringence, image B (superimposed in the upper right corner) as held, visualization in increased 300%.

2.5 Histology

The samples were prepared on pathology laboratory of Odontology College of USP and processed by the histological techniques laboratory of the Institute of Biomedical Sciences (ICB USP/SP).

2.5.1 Processing of samples for histology

Samples of tendon were immersed in buffered solution formaldehyde 10% for fixation. After 48 hours, these tissues were transferred to 70% alcohol:water solution, in volume. This solution was renewed periodically, to removal of fixative residue. The tissue was immersed in a 50% increasing alcohol; water solution, in volume, until absolute alcohol to complete dehydration of the pieces, and later were diaphanized and stained in xylol at room temperature.
The pieces were placed in paraffin, then the 5 µm thick cuts on microtome Leica RM2255 (Leica Microsystems, Welzar, Germany) and stained with hematoxylin-eosin (HE).

3. RESULTS

3.1 Macroscopic Analysis and Optical Coherence Tomography (OCT)

With the macroscopic analysis, it was possible to make an initial assessment of the methods used for sterilization of tissue, noting clear differences between the methods A and B.
The OCT was used to evaluate the morphology of the tissues. By means of this technique it was possible to obtain cross-sectional images of tissues using pulsed light penetrating the tissue without causing damage to the material, and thus evaluate directly your morphology. It can be observed in the sample (method A) (Fig. 2A) the tissue is fit without apparent changes in your structure and (Fig. 2B) in the image processed by the OCT, it can be observed that the method not cause changes to the characteristics like color and tissue structure.

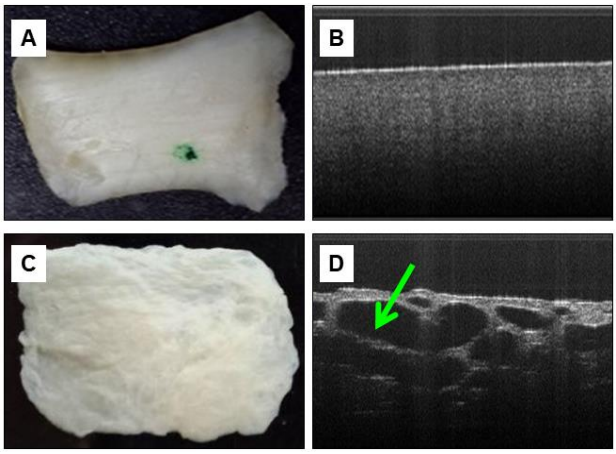

Figure 2: Image A sample processed by method A, on image B sample processed by OCT. Image C sample processed by method B, on image D sample processed by OCT showing changes in tissue structure.

However, in the tissue processed by method B (Fig. 2 C) there was changes to its structures losing completely their characteristics and showing bubbles internalized, that can be best observed in OCT image (Fig. 2 D), confirming the lack of homogeneity of the tissue.

3.2 Analysis of optical coherence tomography Polarization-sensitive (PS-OCT)

The analysis by PS-OCT was used to evaluate the orientation of collagen fibers, which can be identified in the polarized areas of images showed in Fig. 3.

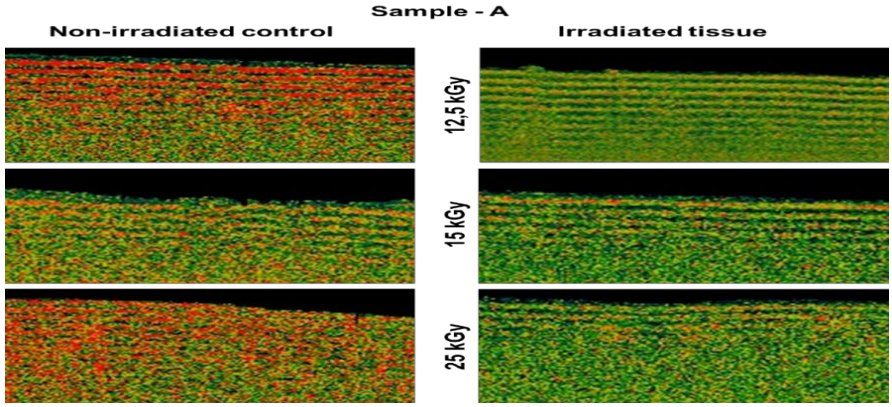

Figure 3: Images obtained by PS-OCT sample to be processed by the method A

It was possible to observe that the tissue processed by the method A observed in Fig. 3 (samples A) presenting homogeneity in their structure, with several birefringence bands in the material, showing the orientation of collagen fibers, as much before as after irradiation.

In the samples processed by method B in Fig. 4 changes were observed in the images of the samples, and there is no homogeneity in your structure, but being able to see some areas in birefringent before and after irradiation of material with their respective doses 12.5, 15 and 25 kGy.

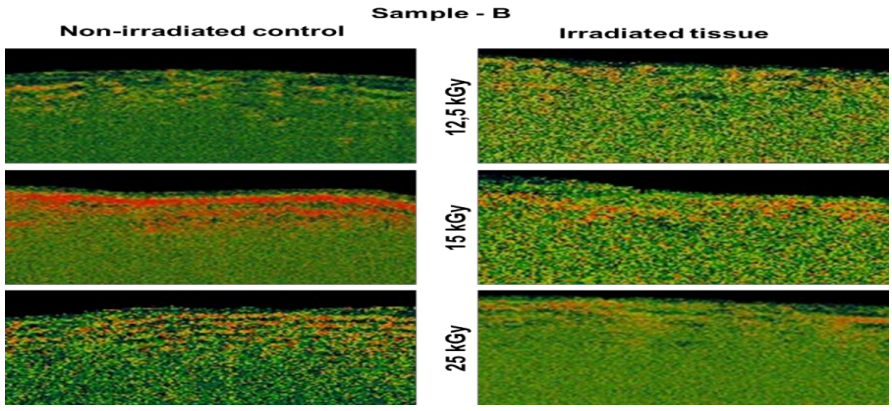

Figure 4: images obtained by PS-OCT sample B processed by method B.

In both forms of preservation and in all doses of radiation can verify the delay phase that shows the presence of birefringence, in this case, the collagen.

In the birefringence image of method A it is possible to observe the bands of fibers in both images (non-irradiated and irradiated), which is not observed in the samples of method B where the sharpness is much lower than both images of non-radiating and irradiated.

3.2.1 Statistical analysis of data

Through the data obtained in Table 1 are for statistical analysis of each group of tendons (irradiated vs non-irradiated) shown in Fig. 5 (where the media were analyzed the distances in between bands of birefringence microns), table 1 shows the average and standard deviation obtained from each sample, comparing the dose groups with their control.

Table 1: Comparison of means values and standard deviation of measurements in micrometers obtained from the bands of birefringence, between control and irradiated group

Dose / Sample	Group control 0 kGy	Group 12.5 kGy	Group 15 kGy	Group 25 kGy
A01	155.1 ± 0.89	167.6 ± 0.55	165.8 ± 0.97	163.8 ± 0.89
A02	157.0 ± 1.03	169.9 ± 0.79	166.9 ± 0.79	162.0 ± 0.90
A03	153.3 ± 1.44	167.4 ± 0.76	162.0 ± 0.74	151.2 ± 0.87
A04	151.0 ± 0.80	161.3 ± 0.98	157.3 ± 1.00	150.7 ± 1.00
A05	168.6 ± 0.71	169.2 ± 0.84	166.4 ± 0.93	157.2 ± 0.94
A06	157.3 ± 0.95	155.0 ± 1.24	151.8 ± 1.26	146.8 ± 0.76
A07	159.5 ± 0.85	169.9 ± 1.45	154.8 ± 1.45	149.3 ± 1.58
A08	144.7 ± 0.64	165.8 ± 0.76	163.6 ± 0.76	161.4 ± 0.84

Values represent the mean ± standard deviation. Statistical significance with ANOVA $p < 0.05$.

The result of the analysis of variance unifactorial (ANOVA), control non-irradiated group versus irradiated group. The Tukey test was applied to show the consistency between the values of the averages.

In Fig. 5 is possible to observe the statistical analysis of the data obtained with the image processing of the samples by the program *ImageJ* through analysis of variance (ANOVA) unifactorial using the program *Graph Pad Prism*, and the results showed varying degrees of correlation.

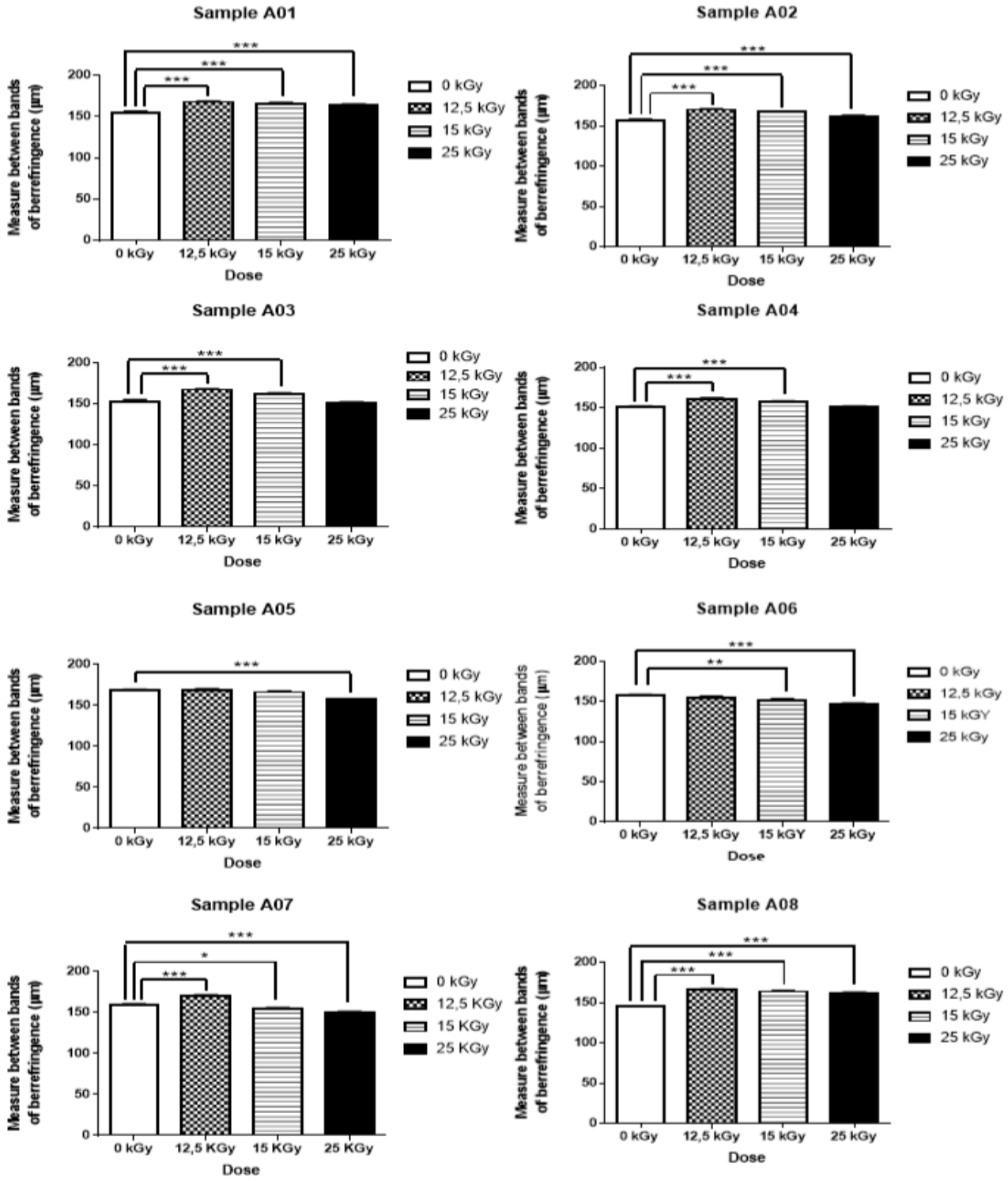

Figure 5: Comparative charts of the averages of distances between birefringence bands, irradiated samples compared to their respective control. Using the analysis of ANOVA and Tukey secondary test, where (*) shows a positive correlation low significance, (**) degree of moderate significance and correlation (***) shows a highly significant correlation.

The samples A01, A02 and A08 showed similar results when related with data of irradiated tendons with their respective controls.

The samples A03 and A04 showed a highly significant correlation with respect to the control and 12.5 kGy and the control and 15 kGy, and showed no significance between control and 25 kGy.
The sample A05 shows highly significant correlation between the control and 25 kGy, but with other samples there was no significance.
The sample showed a highly significant correlation A06 between control and 25 kGy and a moderate relationship between the control and 15 kGy and showed no significance between control and 12.5 kGy.
The sample A07 presented a highly significant correlation between the control and 12. 5kGy and control and 25 kGy, and presented a significant moderate correlation between the control and 15 kGy.

It was possible observe difference only between the irradiated samples at the doses of 12.5 and 25 kGy Fig. 6 (< p 0.05) and the other samples when compared to each other not shown degree of significance, this difference is due to the very small standard deviation obtained between samples (< 0.9%), while the difference between the samples irradiated (A01 until A08) has a variation of 7.2% to 8.6%.

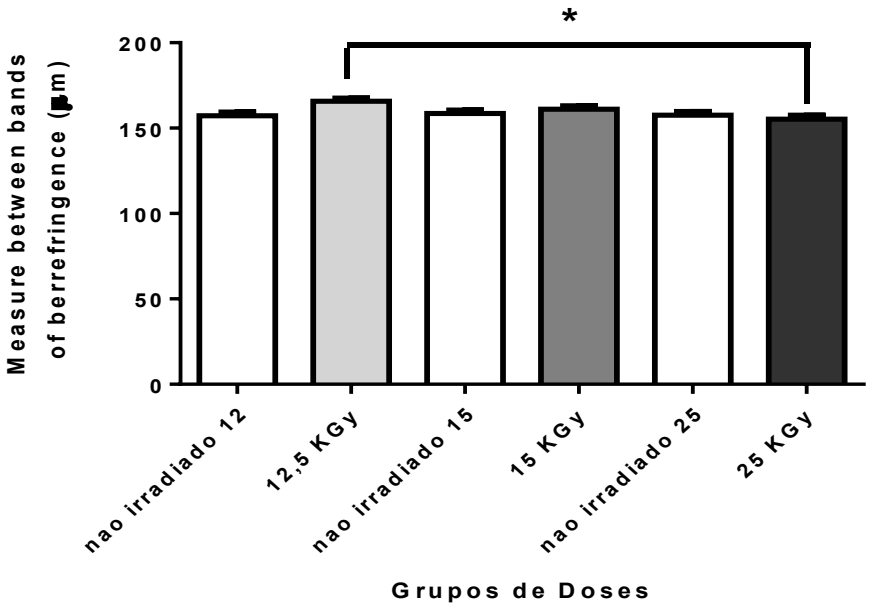

Figure 6: Statistical analysis of the data graph of samples irradiated versus not irradiated, showing the degree of significance between the samples.

Table 2: Comparison of the values of the means and standard deviation of measurements in micrometers obtained from the bands of birefringence's, before and after irradiation of groups

Grupo Controle	12.5 kGy	Grupo Controle	15 kGy	Grupo Controle	25 kGy
157.3 ± 2.12	165.7 ± 1.83	158.7 ± 1.92	161.1 ± 2.03	157.6 ± 2.09	155.3 ± 2.33

Values represent the mean ± standard deviation. Statistical significance with ANOVA $p < 0.05$ (*).

3.3 Histological Analysis

The histological study of the samples was carried out by means of the technique of hematoxylin/eosin staining.

In Fig. 7 an example of the histological appearance of HE-stained tibial tendon samples, observed by light microscopy, in magnification of 100 µm and 200 µm. In the sample A (method A) the non-irradiated tendon (control) presents fibers in different directions of orientation, as is characteristic of this tissue.

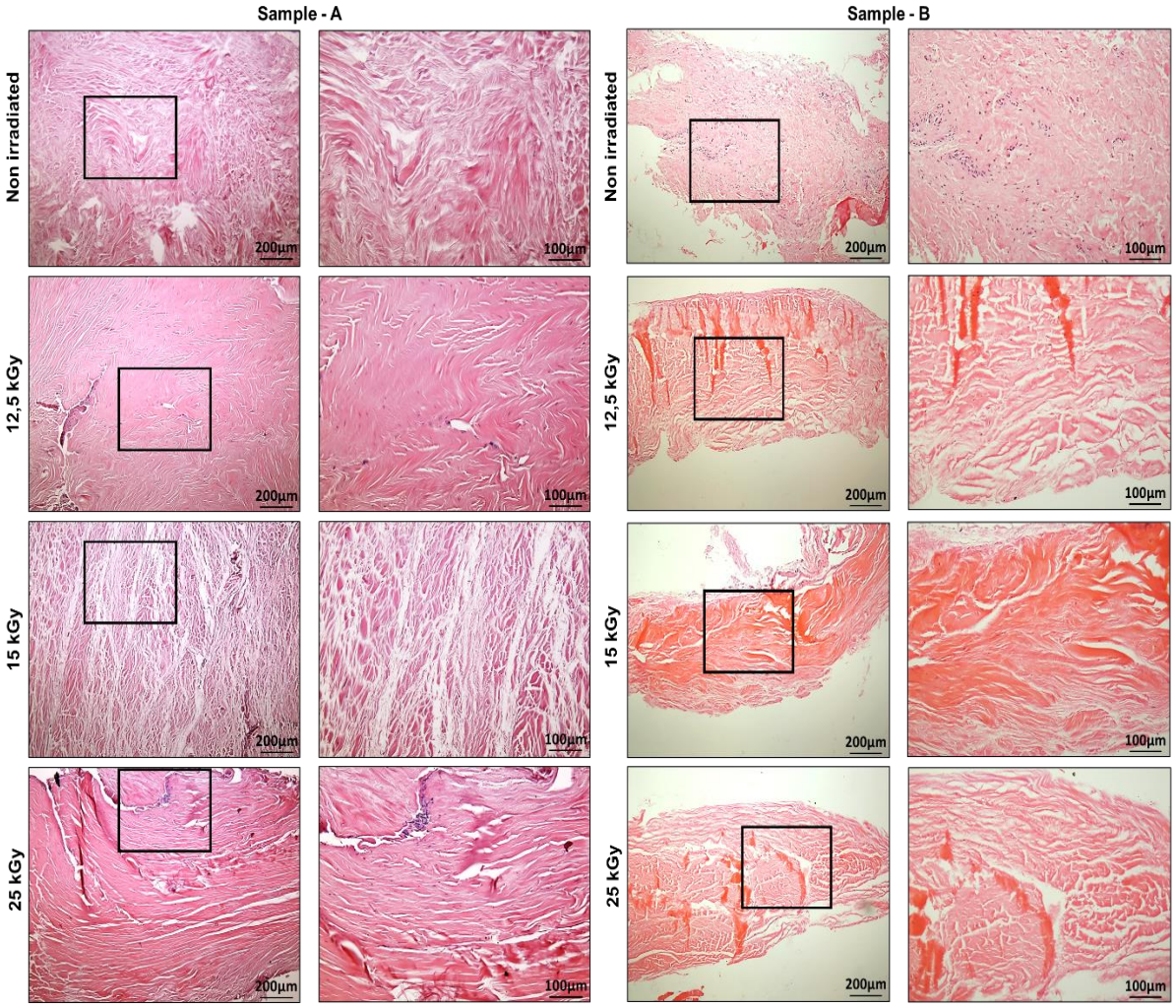

Figure 7: Photomicrography posterior tibial tendon in histological sections showing parallel and transverse beams of collagen fibers (hematoxylin-eosin, 100 µm and 200 µm) control samples, 12.5, 15 and 25 kGy (sample A and B)

Sample gamma irradiated with 12.5 kGy radiation absorbed dose presented a change in the bundles of the tissue appear to be compact, the dose of 15 kGy apparently appears to be similar

to the control and the dose of 25 kGy presented a contraction of the collagen bundles, with spacing greater between fibers.
The hematoxylin/eosin staining shows the tissue morphology, allowing the observation of possible alterations and in none of the samples of method A was observed collagen breakage.
In the sample B (method B) it was possible to observe disorganization in the fibers of the samples and spacings between fibers in all samples, these spaces between fibers increases in agreement with the increase of the radiation dose absorbed by the samples.

4. CONCLUSIONS

Of the two processes used in the preparation of the samples just processing with alcohol and antibiotic proved to be effective for this kind of tendon.
There have been some changes in the morphology of the tissue by ionizing radiation, but the integrity of the collagen fibers was preserved in the dose range studied.
In the radiation doses studied, the collagen fibers orientation was not compromised.

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