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COMPARISON OF TECHNIQUES FOR MORPHOLOGIC EVALUATION OF THE REPAIR PROCESS AFTER TRANSPLANTATION OF GLYCEROL-PRESERVED HUMAN SKIN SUBJECTED TO GAMMA IRRADIATION

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ABSTRACT

Extensive skin lesions expose the body to damaging agents, which makes spontaneous regeneration difficult and, in many cases, leads patient to death. In such cases, if there are no donating areas for autograft, allografts can be used. In this type of graft, tissue is processed in tissue banks, where it can be subjected to radiosterilization. According to *in vitro* studies, gamma radiation, in doses higher than 25 kGy, causes breakdown of collagen I fibrils in the skin preserved in glycerol at 85% and this change influences fibroblast migration and deposition of new collagen. In order to assess if the alterations observed *in vitro*, would compromise *in vivo* use, transplants of human tissue, irradiated or not, were performed in Nude mice. After the surgery the skins of the mice was subjected to macroscopic analysis on the 3rd, 7th, 21st and 90th days; optical coherence tomography on the 90th day and histological assay on the 3rd, 7th, 21st days to compare the results of the repair process among the

techniques, considering that the OCT allows *in vivo* and not destructive morphological analysis. According to the results obtained through OCT it was possible to observe a more organized repair process in the animals which received irradiated grafts (25 and 50 kGy) if compared to unirradiated grafts. It was not possible to observe such phenomena through macroscopic or histological evaluation.

1. INTRODUCTION

Extensive skin lesions expose the body to damaging agents which makes spontaneous regeneration difficult and, in many cases, leads patient to death (Clark et al., 2007). In such cases, if there are no donating areas for autograft, allografts can be used (Barreto, 2006). In this kind of graft, tissues are processed in tissue banks, where ionizing radiation can be used for sterilization (Herson & Mathor, 2006). This method does not result in radioactive residue, does not produce toxic substances, or pose recontamination risk; it can be employed at the temperature the materials are stored, and there is no need for quarantine (Herson & Mathor, 2006). Nonetheless, there might be modifications in the tissue, depending on the type of tissue or irradiation, dose rate, and the dose applied.

According to *in vitro* studies, the effects of ionizing radiation can produce alterations, especially to the skin, such as: functional changes in the stratum corneum, increased diameter of collagen fibrils (Leontiou et al., 1993), change in the architecture (Ottani et al., 2001) and molecular organization of collagen fibrils (Tzaphlidou, 2002), as well as alteration in biomechanical characteristics (Bourroul, 2004). Despite the changes registered in vitro, there have not been published any *in vivo* studies, to demonstrate if such changes in the dermis collagen system may compromise the use of such tissue as allografts.

Also, clinical observations made by Herson[1] (2006) suggest a greater integration of human dermis, preserved in glycerol and submitted to ionizing radiation, with the receptor's tissue, if compared to dermis which has been preserved in glycerol but not irradiated. However, the lack of complementary *in vivo* studies shows the need for further evaluation to confirm such findings.

Therefore, the objective of this study was to evaluate the morphological characteristics of human skin preserved at high concentrations of glycerol and sterilized by a ^{60}Co ionizing radiation source, de-epidermized or not, through macroscopic and histological analysis and by optical coherence tomography, after grafting in nude mice as an experimental model.

2. MATERIAIS E MÉTODOS

2.1. Sample collection and preparation

All the protocols were previously approved by the Committee of Ethics in Animal Research of IPEN-CNEN/SP as well as the Committee of Ethics in Human Research of ICB – USP and

[1] HERSON MR. Clinical use of human tissue allograft preserved in glycerol and irradiatiated. Personal communication [2006].

the Declaration of Helsinki protocols were followed. The procedures described herein were conducted according to the regulations established by Decree CVS-01 of January 18, 2000 (State of São Paulo, Decree CVS-01, 2000).

Human skin samples were obtained from a multiple-organ donor, harvested with an electric dermatome, preserved in concentrated glycerol (minimum 85%) provided by the Tissue Bank of the Central Institute of the Hospital of College of Medicine of University of São Paulo, and were comprised of skin which had been discarded for use in transplants according to Decree number 2.600 of October 21, 2009 (Brazil, Decree number nº 2.600, 2009).

The human skin samples selected were between 0,40 mm and 0,60 mm thick, determined with universal calliper 536 series (Mitutoyo Sul Americana Ltda, São Paulo, Brazil). The samples were measured with a stainless steel ruler (Endo Keiki, Niigata, Japan) and cut in 2,0 x 2,0 cm squares with a blade scalpel number 15 (Solidor, São Paulo, Brazil). After that, the samples were sealed in a double polyethylene package (Whirl-Pak® Write-On Bag, Nasco, Fort Atkinson, Wisconsin, USA) where the radiation indicator seal was placed (WPC Polímeros Ltda, São Paulo, Brazil).

The samples were then irradiated in a ^{60}Co irradiator (Gammacell-220, Nordion Inc., Quebec, Canada) at the Radiation Technology Centre of the Energetic and Nuclear Research Institute (IPEN-CNEN/SP), at a temperature of 2 to 8 ºC, with activity of 3.484,292 Ci and dose rate of de 2,88 kGy/h.

The samples were sorted in three groups: 1) control not irradiated, 2) irradiated with a dose of 25 kGy, and 3) irradiated with a 50 kGy dose.

In order to characterize the repair area after human skin transplant, experiments with both partial skin and human dermis, preserved in high glycerol concentration, irradiated and rehydrated.

To obtain human dermis grafts, after irradiation, some of the skin samples of the experiment groups were washed three times with saline solution at 0,9% for 15 minutes, and subjected to dermoepidermal separation with Dispase solution at 2,5% (Neutral Protease – level II, Boehringer-Mannheim, Germany) in DMEM (GIBCO™, Invitrogen Corporation, California, USA), washed twice for 15 minutes with saline solution at 0,9% and stores in DMEM to which an antibiotic-antifungal (penicillin G sodium 10.000 units/ml, streptomycin sulphate 10.000 μg/ml, amphotericin B 25 μg/ml - GIBCO™, Invitrogen, California, USA) at 4 ºC until use.

2.2. Surgical procedure

Nude mice, both male and females, aged 8 to 10 weeks, supplied by the vivarium of the Biotechnology Centre of IPEN were used. A wound, measuring 1,5 x 1,5 cm, was surgically created, on the dorsal midline of each animal. Animals were randomized into 3 groups, according to treatment. The human skin grafts, whole or de-epidermized, which had been subjected to three 15-minute baths, with sterile saline solution at 0,9%, were kept at surgical sites by Titanium Clips LT100 (Ethicon Endo-Surgery Inc., Cincinnati, Ohio, USA) and fibrin glue (TissueBond®, Recife, Brazil). They were protected against dehydration with Vaseline gauze (Curatec Age 30 Rayon, Johnson & Johnson Medical Inc., New Brunswick, New Jersey, USA) and micropore bandages (3M do Brasil, São Paulo, Brazil) for the whole skin grafts, and polyurethane dressing (Bioclusive, Johnson & Johnson do Brasil Indústria e Comércio para Saúde Ltda, São Paulo, Brazil) for dermal grafts.

2.3. Killing of the animals

The animals were killed in gas chamber (Beira Mar, GSCO2, São Paulo, Brasil) with a coupled carbon dioxide cylinder (White Martins, São Paulo, Brazil). The animals were killed on the 3rd, 7th, 21st and 90th days, according to the procedure for each experiment, and the repair areas were observed and photographed before the removal of the skin for histological analyses and optical coherence tomography.

2.4. Measurement of residual repair areas.

After 21 and 90 days, the residual repair areas were measured. To do so, irregular areas were copied with the aid of transparent paper. In order to gauge the weight and convert it to area, 10 fragments of the paper with an area of 1 cm^2 were cut, those fragments were then weighed in an analytical balance (AB304-S, Mettler Toledo, São Paulo, Brazil), which showed an average weight of 0,0061 g. Other areas were obtained simply by weighing the respective copies and through a simple proportionality equation.

2.5. Removal of mice skin samples

The skin was removed through an incision made with a 15-blade scalpel and dilatation with blunt-tip scissors (Erwin Guth Ltda, São Paulo, Brazil), and kept in saline solution until the optical coherence tomography or cuts for biomechanical assays were conducted. To produce histological slices, the samples were immediately fixed in paraformaldehyde at 4%.

2.6. Optical coherence tomography (OCT)

After the 90th day, two samples from each experiment group were analysed using OCT, as well as uninjured skin from each animal. The equipment used was OCP930SR (Thorlabs, Newton, New Jersey, USA) available at Lasers and Application Centre (IPEN-CNEN/SP). Light was aimed at the sample at a 3-cm focal distance, and the images acquired for both the repair and uninjured areas were stored in a computer and later compared.

2.7. Histology

The skin removed from the repair areas of the animals was fixed in a phosphate-buffered solution containing 4% paraformaldehyde for ≥48 h. Paraffin-embedded tissue was stained with hematoxylin and eosin as a routine stain, and the histological slices obtained were then observed under a light microscope and the pictures were made with a digital camera (DSC-W350, Sony Corporation, Tokyo, Japan), which were then stores in a computer for comparison.

2.8. Statistical treatment and interpretation of data

The data collected were evaluated using Analysis of Variance (ANOVA) in order to assess the differences between the averages of experiment groups, as well as the Tukey test, which compares the lowest difference between averages, using GraphPad Prism 5.0 statistical tool (Graph Pad Software, San Diego, California, USA). The significance level assigned was 5% ($p<0,05$).

3. RESULTS AND DISCUSSION

In vitro deepidermization was conducted in order to obtain dermis grafts. In the control group separation of dermis and epidermis started at 26 minutes and was finished in 50 minutes. In the groups subjected to 25 kGy and 50 kGy it started at 34 minutes, in the 25 kGy group it was finished at 60 minutes, and in the 50 kGy group at 90 minutes. Even after the second incubation, mechanical scraping was necessary to ensure complete dermoepidermal separation in the irradiated groups, and even then, traces of epidermis were found in the irradiated groups, with a greater quantity in the 50 kGy group.

Confirming *in vitro* findings, Herson *et al.* (2001) describe dermoepidermal separation of unirradiated human tissue as occurring between 45 and 60 minutes, proportional to the thickness of dermal fragments.

The greater adhesion, also observed by Paggiaro (2011), in his *in vitro* study with amniotic membrane irradiated with 25 kGy in ^{60}Co, is probably due to changes in the basal membrane. Because of the difficulty of performing deepidermization in irradiated skin samples, it had been hypothesized that the persistence of skin grafts would be a barrier to the migration of mice tissue, warranting comparison of the results with those of dermis grafts.

After transplantation of human skin, the repair area was assessed through macroscopic appearance, and size of the repair area 3, 7, 21 and 90 days after surgery; optical coherence tomography images were obtained after 90 days and histological evaluation after 3, 7 and 21 days. These times were based on the phases of repair process that occur on the animals’ skin.

3.1. Macroscopic evaluation

Post-surgical wounds were evaluated for all experiment groups, as well as the control group (Figure 1). After 3 days, some wounds displayed little exudate, but most were dry in all experiment groups. After 7 days, the edges of the wounds had dried up in all experiment groups and, until that moment, there were no clear differences in the groups evaluated. After 21 days, all wounds presented with dry, scaly crusts and wound area was smaller, especially the 25 kGy group. After 90 days, a continuous thin epithelial layer was formed with the closing of wounds.

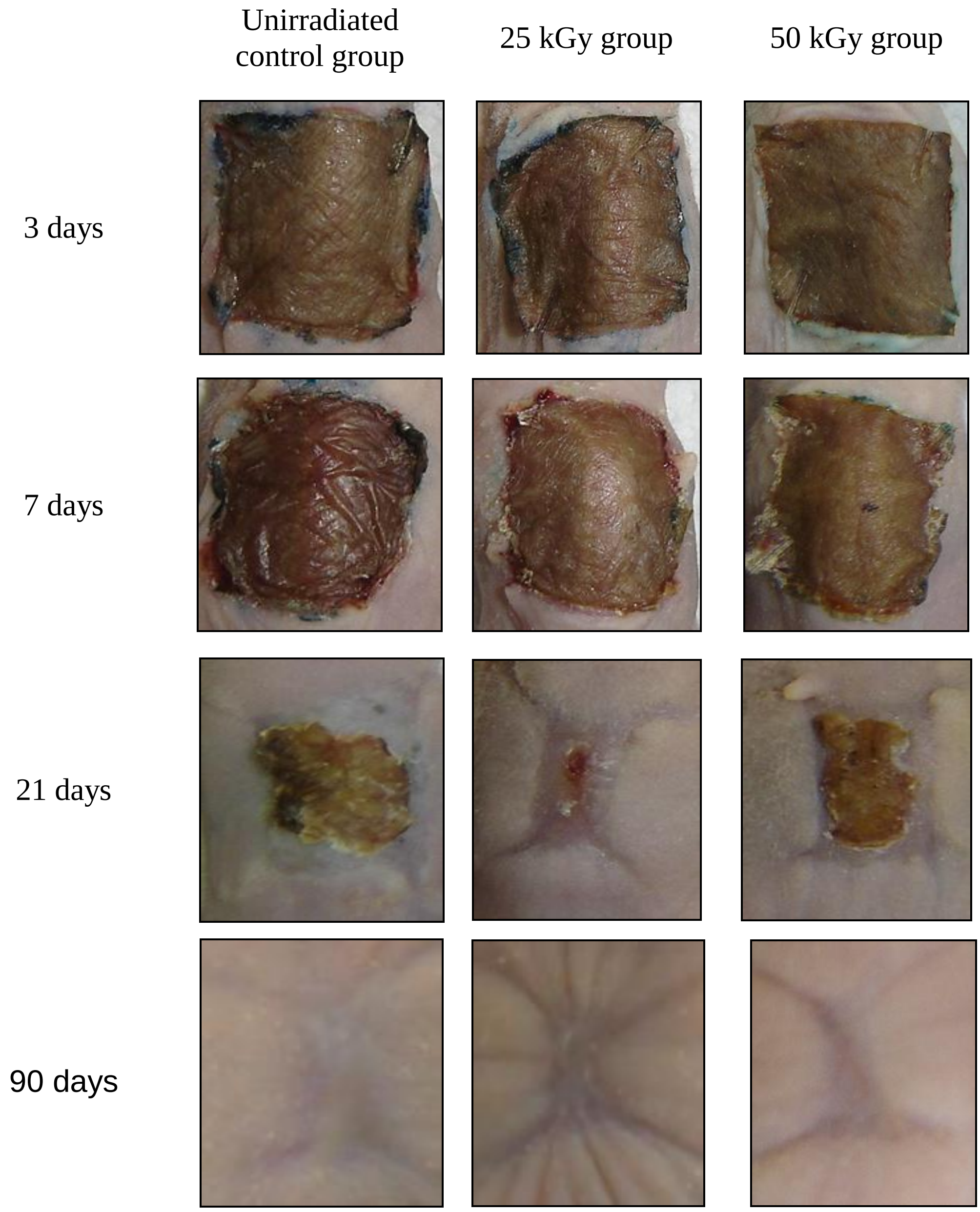

FIGURE 1 – Macroscopic aspect of wound and repair areas in experiment group animals 3, 7, 21 and 90 days after human skin transplantation.

Repair areas were measured after 21 and 90 days. After 21 days, an area of lesser tissue reparation and greater contraction was observed in the animals that received human skin grafts irradiated with 25 kGy, resulting in disfigurement. Values showed statistical significance when compared to control groups (34,00 ± 0,58%) x 25 kGy (27,25 ± 1,61%) and 25 kGy group (27,25 ± 1,61%) x 50 kGy (34,88 ± 2,58%) (Figure 2a), confirming a smaller repair area in the 25 kGy group.

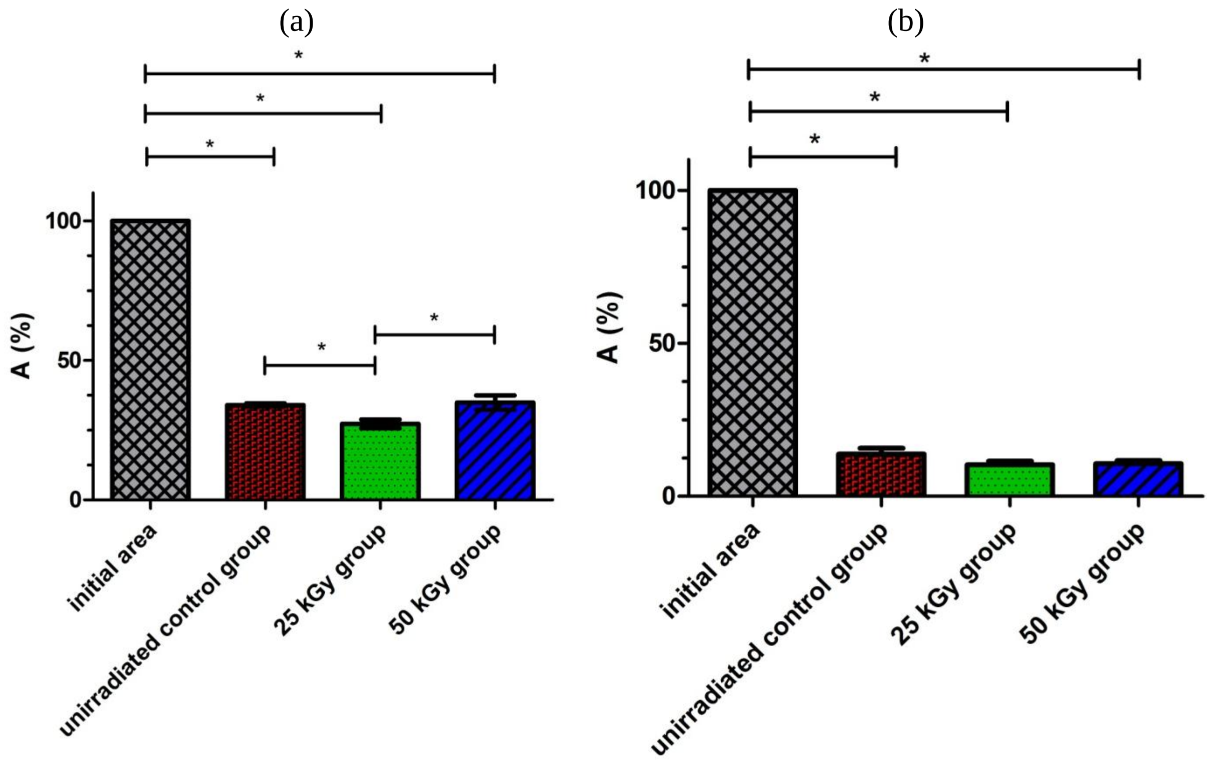

FIGURE 2 – In (a) is a graphic representation of the values of repair area (A) 21 days after the human skin transplantation. Results are shown as averages (± SEM). Value: *p<0,05. N = 5 per group. In (b) the values of repair area 90 days after the human skin transplantation are shown. Value: *p<0,05. N = 8 per group.

After 90 days, all groups displayed a smaller repair area and, even though there were no statistically significant differences among them (Figure 2b), it was possible to discern greater contraction and smaller tissue reparation in the animals which received irradiated skin grafts. In the group irradiated with 25 kGy such effects were apparently more intense (Figure 1).

3.2. Evaluation with optical coherence tomography images

In order to evaluate the organization of the repair process samples were analyzed, using OCT, in relation to light scattering homogeneity and the distinction between epidermis and dermis. The differences between uninjured skin and experiment groups are shown in Figure 3. It was possible to observe a greater distinction between the epidermis and dermis in the region that received irradiated grafts. The animals that received irradiated grafts with 50 kGy showed scar area with greater light scattering, greater homogeneity, greater distinction between the epidermis and dermis, indicating a better repair.

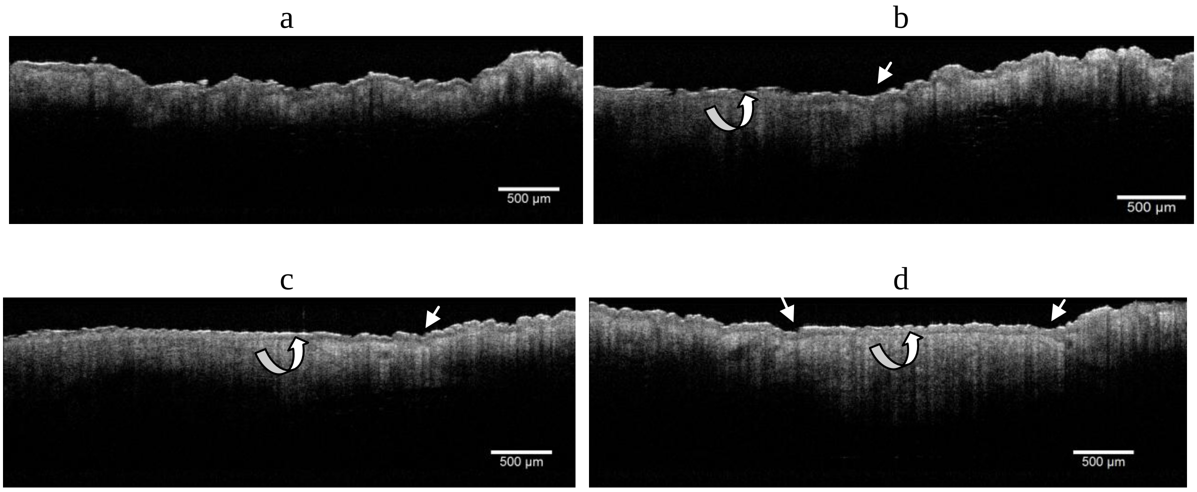

FIGURE 3 – OCT Images of Nude mice. In (a) uninjured Nude mice skin, (b) Nude mice skin subjected to transplantation with unirradiated human skin, (c) Nude mice skin subjected to transplantation with human skin irradiated at 25 kGy, (d) Nude mice skin subjected to transplantation with human skin irradiated at 50 kGy, (↙) interface area between Nude mice skin x repair area after human skin transplant, (↻) distinction between dermis and epidermis.

However, in the group that received unirradiated grafts, the interface between the repair area and the original mice tissue was less homogeneous (Figure 3b).

Although it shows less microstructural details than light microscopy, due to the multiple light scattering and the limitation in image depth (Fujimoto *et al.*, 1995), the results obtained with OCT can be compared to those obtained with light microscopy at a depth of approximately 600 μm (Pan *et al.*, 1996), having the advantage of functioning as a kind of "optical biopsy" where the morphology can be assessed directly, in real time and without any prior preparation of the structures to be studied (Fujimoto *et al.*, 1995).

3.3. Evaluation with light microscopy of histological sections of mice skin where human graft transplants were conducted

Through histological analysis of the skin of mice, which were killed after 3 days, it was possible to detect the presence of intense inflammatory infiltrate, little tissue organization, without clear differences between the groups (Figure 4). After 7 days, it was possible to observe migration of mice epidermis onto the probable human dermis only in one of the edges in 60% of the slides in the control group (not irradiated), whereas for the group irradiated with 25 kGy, the migration occurred in both edges in 54,5% of the slides, and for 50 kGy group, in one of the edges of one slide only (Figure 4). Such difference in mice tissue migration behaviour was initially related to the presence or absence of epidermis in the graft, as in irradiated groups dermoepidermal separation was harder, especially in the 50 kGy group.

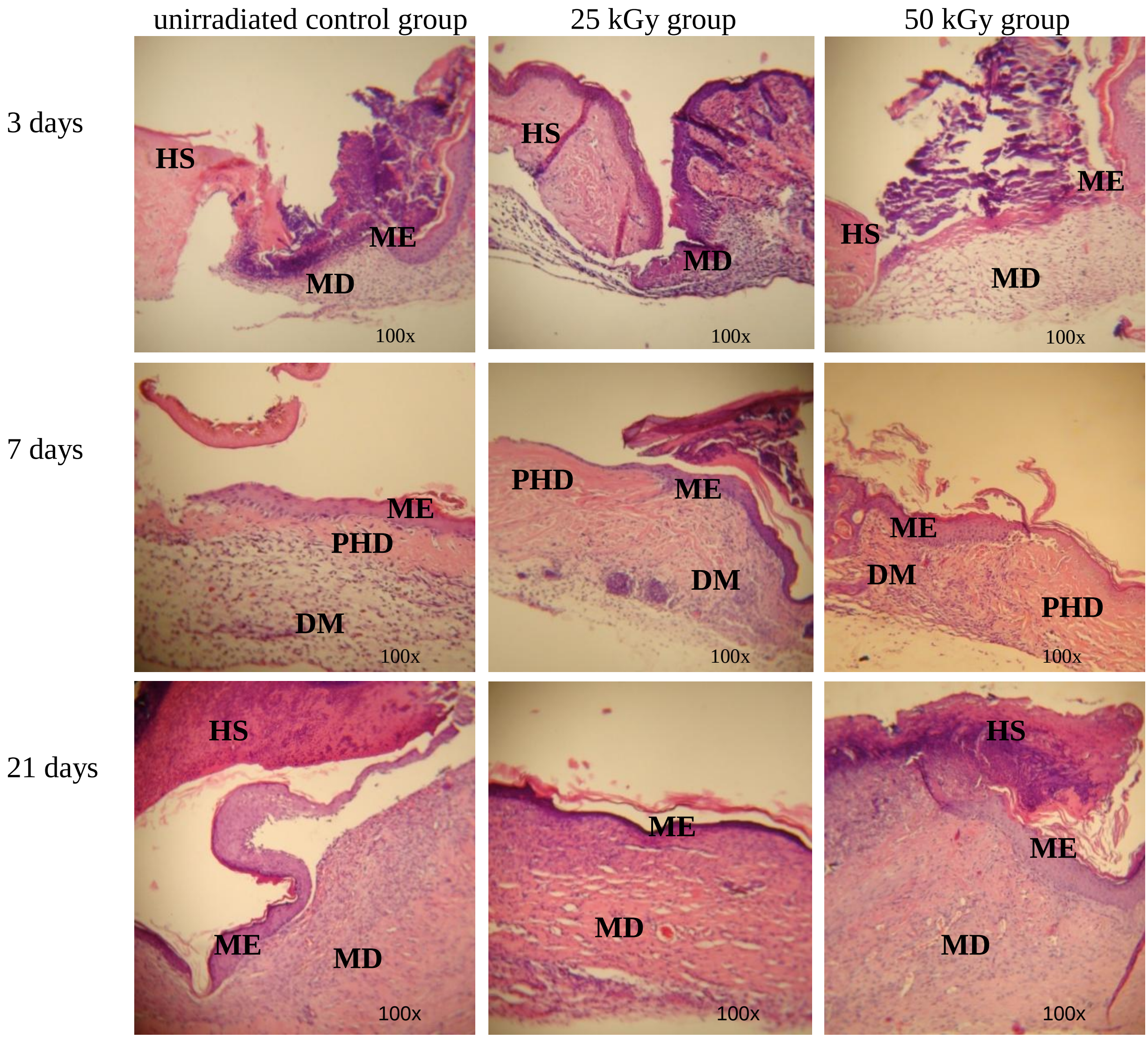

FIGURE 4 - Nude mice skin with human tissue graft at periods varying from 3 to 21 days, for doses of 25 kGy, 50 kGy and unirradiated control – Light microscopy (HE stain). Mice epidermis (ME), mice dermis (MD), human skin (HS), probable human dermis (PHD). Magnification: 100x.

After 21 days, the wounds where grafts irradiated with 25 kGy were used closed more quickly if compared to the unirradiated group. In the group exposed to 50 kGy, wounds did not close (Figure 4). This characteristic observed in the 50 kGy group could be related to the effects of radiation, as described by *in vitro* studies (Gouk *et al*., 2008), and due to a slower reparation, deposition of new collagen was more organized, thus resulting in better reparation.

If these findings are extrapolated to human skin, then 25 kGy gamma radiation would cause tissue changes which promote the conditions for coverage, faster closing and collagen synthesis, as well as migration of cells into the wound bed.

3.4. Morphologic characterization of human dermis transplanted onto Nude mice

In order to verify if the presence of dermis would make the migration of mice tissue more difficult, human tissue (irradiated or not) transplants were conducted and the animals were killed after 7 days.

In the present experiment it was possible to observe an intense inflammatory process (data not shown) and, in all of the groups studied, mice epidermis migrated under human dermis towards the centre of the wound (Figure 5).

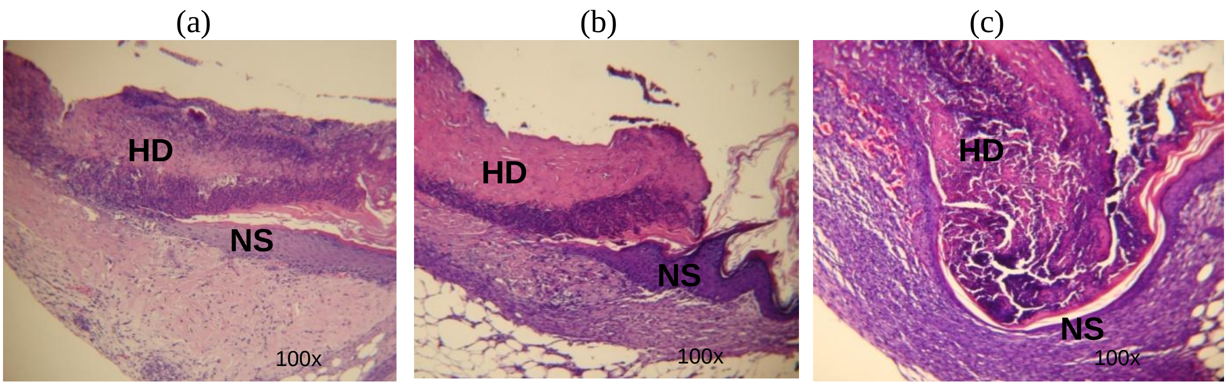

FIGURE 5 - Nude mice skin (NS) with human dermis graft (HD) - Light microscopy. Killed 7 days after receiving human dermis graft. In (a), unirradiated control group; in (b), 25 kGy group and in (c), 50 kGy group. Magnification: 100x.

Even though, at first it was hypothesized that the permanence of human epidermis was a barrier for the migration of mice tissue, this was not confirmed with the use of human dermis.

Even though the animal model used in the present study has immunologic behaviour which is different from humans, Nude mice are described in the literature as the animal of choice for studies of skin repair after human graft use (Mecklenburg *et al.*, 2005, Gawronska-Kozak *et al*, 2006), and enables the extrapolation of some of the results obtained to humans, mainly in comparative studies, such as the response of irradiated in comparison to unirradiated grafts.

4. CONCLUSIONS

- Macroscopically the grafts irradiated at 25 kGy promoted greater initial contraction after 21 days, with no difference regarding final repair area dimensions among Nude mice experiment groups after 90 days.

- According to the results obtained through OCT it was possible to observe a more organized repair process in the animals which received irradiated grafts (25 and 50 kGy) if compared to unirradiated grafts. It was not possible to observe such phenomena through macroscopic or histological evaluation.

- According to histological observation, with the use of grafts irradiated at 25 kGy, there was a reduction in wound closing time, in comparison to the control and unirradiated groups, whereas with grafts irradiated at 50 kGy, an inverse effect was observed, as the wounds did not close after 21 days.

- Through histological comparisons among mice that received human skin or dermis grafts, irradiated or not, it was possible to conclude that the absence of epidermis did not help the integration of the graft.

- When grafts irradiated at 25 kGy were used, a comparative study of irradiated and unirradiated grafts, showed an acceleration of the repair process.

- Even though Nude mouse is one of the most used *in vivo* models, its repair response differs from that of human adults, which makes it necessary for these findings to be confirmed in an animal model whose response is similar to humans'.

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