SCIENTIA PLENA
www.scientiaplena.org.br
VOL. 19, NUM. 07 2023
doi: 10.14808/sci.plena.2023.077501

Irradiated peripheral blood mononuclear cells for assessing individual radiosensitivity in laryngeal cancer radiotherapy

Células mononucleares do sangue periférico irradiadas para avaliação da radiossensibilidade individual em radioterapia do câncer de laringe

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(Recebido em 06 de dezembro de 2022; aceito em 05 de julho de 2023)

In laryngeal cancer radiotherapy (LCRT), dermatitis, dysphagia, and odynophagia are common acute side effects, varying in intensity depending mainly on the patient's radiosensitivity. This study investigated the survival rate of in vitro irradiated peripheral blood mononuclear cells (PBMC) as a pre-therapeutic test to assess individual radiosensitivity and predict the degree of these acute side effects in LCRT. 16 patients diagnosed with early-stage laryngeal cancer undergoing radiotherapy without adjuvant chemotherapy participated as volunteers in this study. Before the beginning of treatment, a sample of PBMC was collected and irradiated with 2.5 Gy using a 6 MV linear accelerator. After 72-h cell incubation, the trypan blue exclusion test was performed to score viable cells through optical microscopy. The percentages of cell viability were compared to the intensity of acute side effects experienced by LCRT patients. Even though no correlation was found regarding the observed degrees of radiation dermatitis, our results indicate that patients with survival rates of in vitro irradiated PBMC higher than 70% are more likely to experience moderate to severe acute odynophagia and dysphagia after LCRT. The proposed and discussed methodology points to a practical and affordable pre-therapeutic test to evaluate individual radiosensitivity in order to prevent or reduce acute side effects in laryngeal cancer radiotherapy.
Keywords: laryngeal cancer, radiotherapy, radiosensitivity.

Na radioterapia do câncer de laringe (LCRT), dermatite, disfagia e odinofagia são efeitos colaterais agudos comuns, cuja intensidade varia dependendo principalmente da radiossensibilidade do paciente. Este estudo investigou a taxa de sobrevivência de células mononucleares de sangue periférico irradiadas in vitro (PBMC) como um teste pré-terapêutico para avaliar a radiossensibilidade individual e prever os efeitos colaterais agudos em LCRT. 16 pacientes diagnosticados com câncer de laringe em estágio inicial submetidos à radioterapia, sem quimioterapia adjuvante, participaram como voluntários neste estudo. Antes do início do tratamento, uma amostra de PBMC foi coletada e irradiada com 2,5 Gy usando um acelerador linear de 6 MV. Após 72 h de incubação das células, o teste de exclusão com azul de tripan foi realizado para quantificar as células viáveis por meio de microscopia óptica. As porcentagens de viabilidade celular foram comparadas com a intensidade dos efeitos colaterais agudos experimentados pelos pacientes submetidos a LCRT. Embora nenhuma correlação tenha sido encontrada em relação aos graus observados de dermatite por radiação, nossos resultados indicam que pacientes com taxas de sobrevivência de PBMC irradiadas in vitro, superiores a 70%, têm maior probabilidade de apresentar odinofagia/disfagia de moderada a grave após LCRT. A metodologia proposta e discutida neste trabalho aponta para um teste pré-terapêutico prático e acessível para avaliar a radiossensibilidade individual a fim de prevenir ou reduzir os efeitos colaterais agudos na radioterapia do câncer de laringe.
Palavras-chave: câncer de laringe, radioterapia, radiossensibilidade.

1. INTRODUCTION

Laryngeal cancer (LC) is the most common malignancy among head and neck tumors, being the second type of cancer of the aerodigestive tract, behind lung cancer. This disease is intimately related to patient smoking and alcohol habits. Both habits can cause a synergistic effect, increasing this malignancy risk [1-3].

In the early-stage LC, radiotherapy (RT) is the main treatment modality, contributing to the maintenance of laryngeal physiological functions [4]. RT induces lethal lesions in malignant neoplasias, through ionizing radiation (IR) interaction, generally without causing severe reactions to healthy (normal) tissue adjacent to the tumor. Acute side effects occur within 90 days after initiating the treatment due to healthy tissue reaction to IR, but acute severe ones may compromise the RT outcomes [5]. Radiation side effects are associated with severe adverse skin reactions that lead to dermatitis with dry or moist desquamation, dysphagia (difficulty in swallowing), odynophagia (pain when swallowing), hoarseness, airway obstruction, fistula, and, in rare cases, necrosis [6-8].

Most differences in patients' radiosensitivity come mainly from individual genetic makeup [9]. This phenomenon was first described in patients with genetic syndromes like Fanconi's Anemia and Ataxia-Telangiectasia since these patients have shown severe adverse effects due to their difficulty in repairing DNA radioinduced damages [10]. However, some studies pointed out significant individual radiosensitivity cases that were not associated with any known genetic syndromes [11].

Patients submitted to the same radiotherapy protocol have shown a variation in the degree of side effects in healthy tissue, even though they had similar tumors and the same cancer staging, and almost 10% of patients undergoing RT show serious reactions in healthy tissue, compelling them to interrupt the treatment [12]. Several studies have proposed potential biomarkers analyses of individual radiosensitivity based on cellular or molecular techniques, and most of them are either laborious or high-cost techniques. Although promising, no pre-therapeutic radiosensitivity test is commonly used in oncology practices [13-16].

In this context, this study was designed to evaluate the survival rate of in vitro irradiated peripheral blood mononuclear cells of LC patients undergoing RT as a pretreatment test to assess individual radiosensitivity by correlating the percentage of viable PBMC with the severity of acute adverse effects experienced by early-stage LC patients assigned to radiotherapy.

2. MATERIALS AND METHODS

2.1 Ethical considerations

The Ethics Committee of IMIP (Professor Fernando Figueira Integral Medicine Institute-Recife, Brazil) approved this study, under number registration nº 1,939, which was conducted on the principles of the Helsinki Declaration and voluntary participation: all patients were informed about the purpose of the research, after which written consent was obtained. All recommendations from the Ethics Committee were followed.

2.2 Study group

This study involved 16 patients diagnosed with LC in early stages (T1, 2-N0), according to the TNM staging system established by the Union for International Cancer Control (UICC) [17], 13 males (mean age 62 years; range 49 – 81) and three females (mean age 61 years; range 56 – 69).

All patients were treated with three-dimensional (3D) conformational radiation therapy at IMIP, without adjuvant chemotherapy, using a 6 MV Electron Linear Accelerator (SIEMENS-Primus, USA), receiving 28 (T1) or 29 fractions (T2) of 2.25 Gy/fraction to a total dose of 63 or 65.25 Gy.

2.3 Sampling and in vitro irradiation

6 mL of peripheral blood samples were collected and divided into two syringes of 3 mL from each patient. One blood aliquot was used as a control (non-irradiated). The syringe containing

the other aliquot was placed in a phantom (ρ = 1.0 g.cm^{-3}), a device composed of square blocks of varying thickness and designed to simulate soft human tissue over the range of radiation energies used in radiation oncology, in order to receive a radiation dose of 2.5 Gy (dose rate 2 Gy.min^{-1}), from the same source used for the RT (i.e., 6 MV Electron Linear Accelerator) (Primus K Simens, Concord, USA). This radiation dose is around the daily fraction of the RT protocol of all patients.

2.4 Cell Culture

After the sample irradiation, peripheral blood mononuclear cells (PBMC) were separated by centrifugation (400 g for 35 min) using Ficoll Paque Plus (density 1.077 g.mL^{-1} – GE Healthcare, USA). After washing in phosphate-buffered saline (PBS 0.1 M, pH: 7.2 – 7.4), PBMC were then re-suspended in RPMI -1640 medium supplemented with 10% fetal calf serum, 2 mML-Glutamine, 50 mg.L^{-1} of sulphate gentamicine, and 2 mg.L^{-1} of amphotericin B (Cultilab, Campinas, Brazil) at the density of 2x10^{6} cells.mL^{-1}. The cell suspension was transferred into multi-well culture plates (Sigma-Aldrich, Trasadingen, Switzerland), at a density of 2x10^{5} cells/well, and plates were incubated for 72 h in a 5% CO_2 air-humidified atmosphere at 37°C.

2.5 Trypan Blue Exclusion Test - Scoring of cell survival

Following 72 hours of incubation, 10 µL of cell suspension were put in 90 µL of the trypan blue dye 0.4% (Sigma-Aldrich, St. Louis, USA) at 1:9 dilution (1 part of cells: 9 parts of dye). The trypan blue (TB) exclusion test is based on the principle that intact membranes in live (viable) cells exclude TB dyes, whereas dead cells do not. A hematocytometer (Neubauer chamber) was used to score viable and nonviable cells by optical microscopy. Hence, cell viability (CV) was calculated as follows:

CV (%) = (VC / TC) x 100; where CV (%) = percentage of cell viability; VC = total viable cells (unstained); TC = total cells (viable plus nonviable cells). Mean values of CV were obtained from three biological replicates. The main steps of this procedure are summarized in Figure 1.

2.6 Follow-up of side effects

The monitoring of the side effects of patients was registered every 15 days up to four weeks after the last radiotherapy session. The grading of adverse effects, skin reactions, and swallowing dysfunctions was adapted from the acute radiation morbidity scoring criteria classification of the RTOG-Radiation Therapy Oncology Group [18], as follows: none (grade 0); mild (grade1); moderate (grade 2) and severe (grades 3 and 4).

All subjects were oriented to wash their necks with water and soap and to use a moisturizing cream. Provided by the hospital, all patients received DERSANI™ (Saniplan, Brazil), a cream mainly containing fatty acids and antioxidant vitamins A and E, to be applied twice a day on the irradiated region of the neck. The skin reaction levels were independently evaluated by four physicians (oncologists).

Patients were asked to rank these symptoms through a questionnaire comparing before and after radiotherapy to monitor dysphagia and odynophagia. The patients themselves could not distinguish between dysphagia (swallowing difficulty) and odynophagia (painful swallowing), so they scored the same RTOG grades for both adverse effects. Hence, in this study, the symptoms of dysphagia and odynophagia were grouped as "laryngeal dysfunctions".

In this study, an adverse effect was considered as a) mild if the patient reported no difference or some difficulty in swallowing food compared to before RT; b) moderate if the patient could not swallow all kinds of food, even though they could eat solid food; and c) severe if the patient could not eat solid food.

2.7 Statistical analyses

Spearman's test was performed to examine possible correlations between cell viability and the intensity of side effects. All analyses considered a significance level of 5% and were carried out using the software BioEstat (Version 5.3 version, Instituto Mamirauá, Brazil).

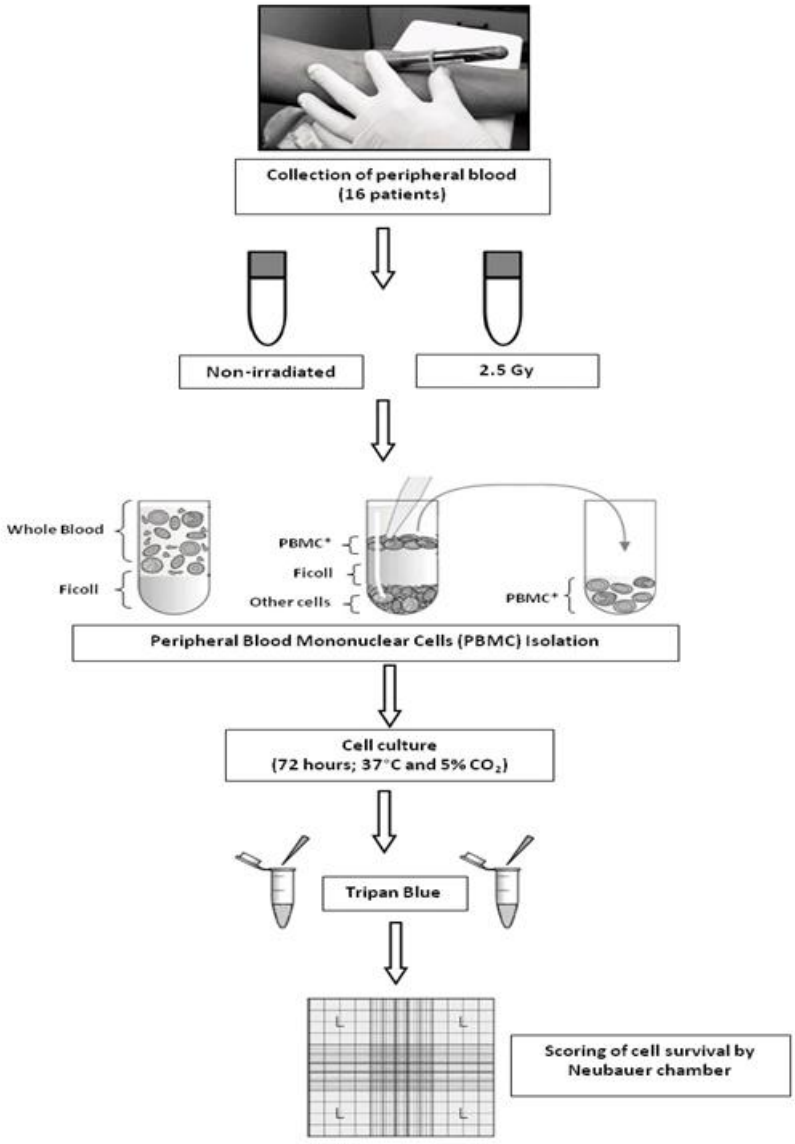

Figure 1: The method used to evaluate the irradiated peripheral blood mononuclear cell survival.

3. RESULTS AND DISCUSSION

This research focused on studying patients diagnosed with laryngeal cancer at the initial stages, without adjuvant chemotherapy, and who received all care at the same radiotherapy center. During the study period (one year), 16 patients met these inclusion criteria.

Figure 2 shows examples of mild (A), moderate (B), and severe (C) degrees of skin side effects from patients studied in this work, classified by the medical board's assessment.

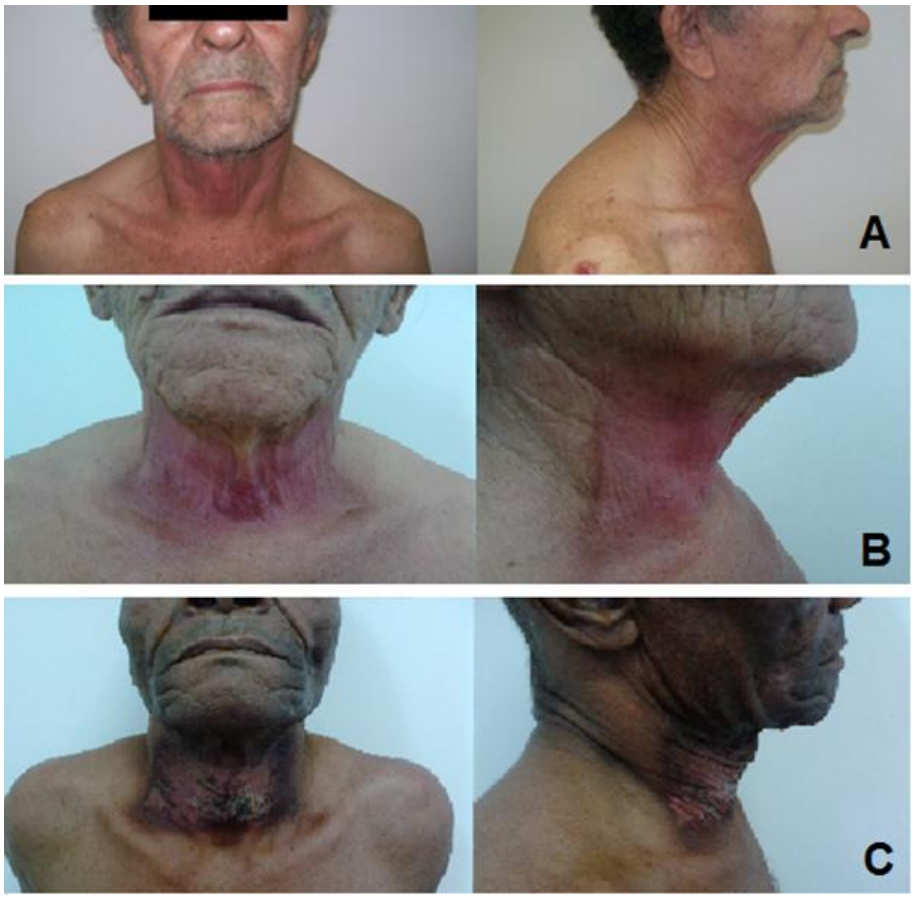

Figure 2: Examples of degrees of skin side effects found in this study: ***(A)*** *mild,* ***(B)*** *moderate, and* ***(C)*** *severe.*

The role of topical control in preventing acute dermatitis was emphasized during the follow-up of patients. In the last session of RT, one patient had his skin reaction graded as mild. One week later, the radio-induced dermatitis of his neck progressed into severe status, presenting moist desquamation. Despite medical recommendations, this patient reported that he had stopped using the moisturizer just after the end of his treatment (Figure 3). This fact is discussed later in this paper.

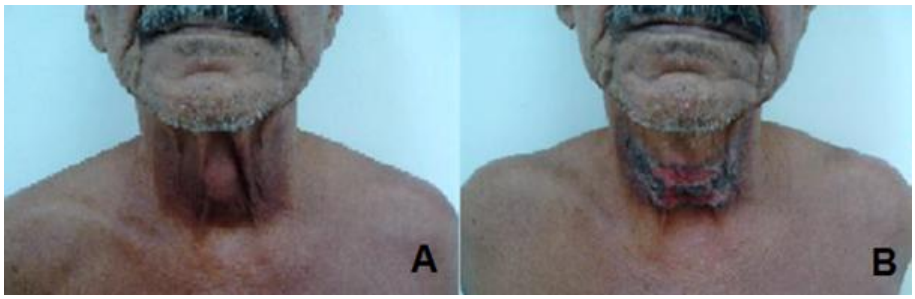

Figure 3: ***(A)*** *Aspect of a patient's skin reaction just at the end of radiotherapy;* ***(B)*** *Dermatitis in the same patient one week after the last session of radiotherapy (without topical control).*

3.1 Cell survival, dermatitis, and laryngeal dysfunctions

After 72 h of incubation, the percentage of non-irradiated PBMC was 97.50 (± 3.40) % for all control samples.

The normality test indicated that the data of laryngeal dysfunctions follow a normal distribution. Concerning the in-vitro irradiated samples, on Spearman's rank correlation coefficient (ρ), no correlation (ρ = 0.1918, p-value = 0.4768) was found between the survival rate of irradiated PBMC and dermatitis.

Table I shows the percentage of survival rate of PBMC from each patient and the respective reported degree of laryngeal dysfunctions based on the classification of acute radiation morbidity scoring criteria defined by the RTOG-Radiation Therapy Oncology Group [18].

Table 1: Survival rates of in vitro irradiated PBMC and the individual RTOG ranking for laryngeal dysfunctions.

Patient	PBMCs Survival (%) *	RTOG Ranking **
1	49.28 (± 0.79)	0
2	53.55 (± 1.90)	0
3	54.30 (± 2.77)	1
4	56.98 (± 1.35)	1
5	58.49 (± 0.97)	2
6	60.14 (± 1.70)	1
7	62.76 (± 2.79)	2
8	64.06 (± 1.76)	1
9	69.17 (± 0.77)	1
10	70.28 (± 1.96)	2
11	72.33 (± 2.34)	3
12	72.90 (± 1.57)	2
13	74.09 (± 1.10)	2
14	74.89 (± 0.29)	2
15	76.47 (± 2.55)	2
16	82.88 (± 3.28)	3

*Mean (± standard deviation).
**No effect = 0; Mild = 1; Moderate = 2; Severe = 3. RTOG – Radiation Therapy Oncology Group.

Figure 4 summarizes the results of Table I according to the RTOG classification related to laryngeal dysfunctions. Spearman's rank correlation coefficient (ρ) indicated a significative correlation (r = 0.7909, p-value = 0.0003) between irradiated PBMC survival and the effects of laryngeal dysfunctions.

The tolerance of healthy (normal) connective tissue to IR is essential for radiotherapy effectiveness [14, 19]. Thus, all efforts to establish a pre-therapeutic test to predict normal tissue radiosensitivity are necessary to provide personalized radiotherapy, avoiding or mitigating side effects [20].

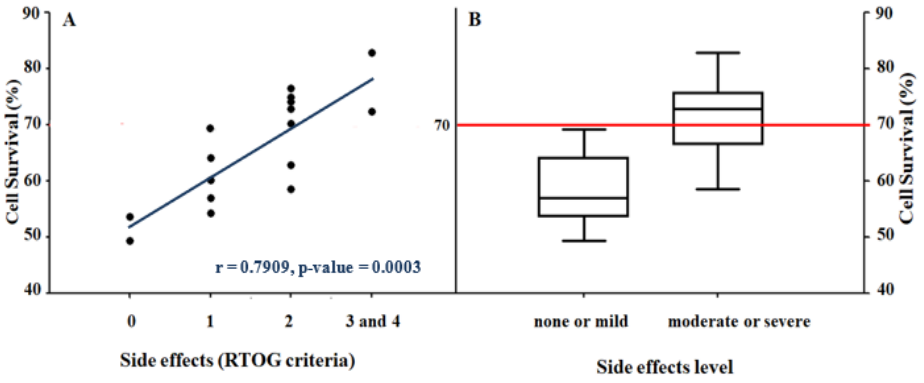

*Figure 4: Graphic representations of cell survival against side effects of laryngeal dysfunctions emphasizing: **(A)** Spearman's rank correlation; **(B)** A red line highlighting that cell survival rates greater than 70% are associated with subjects presenting moderate or severe side effects levels.*

Although several factors influence the development of acute side effects, it has been estimated that most of the variation in normal tissue reactions between patients is likely genetic [21]. As a biological model, peripheral blood is considered an accessible resource for studying genotoxicity and estimating radiation effects on radiation cellular response investigation. Thus, blood-based assays with peripheral mononuclear blood cells (PBMC) have been the most widely employed model due to radiosensitivity, accessibility, and high concentrations in whole blood [22].

Among several methods for evaluating cytotoxicity in experimental investigation, the trypan blue (TB) exclusion test is currently one of the most widely used [23-25]. The relative number of dead and live cells is conventionally obtained by optical microscopy using the Neubauer chamber, one reason for characterizing the TB method as an easy-to-reproduce, low-cost, and rapid assay.

As performed in this study, patients' blood samples could be irradiated in the hospital RT department and sent to its own or nearby cytogenetics laboratory for cell survival analyses. Based on the methodology proposed here, interlaboratory fluctuations regarding PBMC survival percentages may be expected. These variations may arise from laboratory environmental conditions, choice of reagents, handling procedures, and equipment. However, trypan blue staining is the most well-known and affordable test for cell viability studies, a feature that facilitates the implementation of the proposed assay.

3.2 Dermatitis

Regarding the results concerning dermatitis, naturally, patient self-care is essential in managing radiotherapy-induced skin reactions; maintaining adequate skin hydration, which is critical for healthy skin, positively affects skin tolerance to the treatment [26, 27]. As far as we know, no research report shows a strong correlation between topical controls and acute radiation dermatitis among patients undergoing radiotherapy with head and neck cancer, perhaps due to patient self-care representing a significant confounding factor.

Even though all patients were advised to use the moisturizer in this study, ensuring they proceed as recommended is not straightforward. The event here reported, about one patient who interrupted the use of the moisturizer right after the end of the radiotherapy with a reflection on the rapid progress in the grading of the adverse effects on his skin (Figure 3), is an example of the importance of the prophylactic management of skin to prevent radio-induced dermatitis. Thus, it is possible to infer that any occasional misuse of the moisturizer could have influenced the other subjects' skin side effects.

There is currently no standard protocol for preventing and treating radio-induced skin reactions. Several studies have evaluated topical steroids, but randomized controlled trials have not yet consistently emphasized any single agent [11, 26, 27]. However, maintaining proper skin hygiene, i.e., by gentle washing with lukewarm water, mild soap, and unscented, lanolin-free water-based moisturizers, can prevent radiation-induced skin reactions [28, 29].

Several factors may influence the severity of skin reaction to RT, such as the volume of treated region, daily fraction and total doses, tumor stage, adjuvants therapies, social habits (mainly smoking), and genetic history [11]. Due to the small number of subjects, our results concerning dermatitis should, naturally, be interpreted with caution.

3.3 Laryngeal dysfunctions

Considering Table I and Figure 4, and the patients with a survival rate of irradiated peripheral blood mononuclear cells (PBMC) below 70 % (nine patients), the majority (seven patients) ranked the symptoms of laryngeal dysfunctions as being none or mild, while only two ranked as moderate. On the other hand, the other seven patients who presented PBMC survival rates higher than 70% experienced moderate or severe acute side effects. This correlation between the survival rate of irradiated PBMC and normal tissue toxicity of patients undergoing radiation cancer treatment follows previous studies [30, 31].

Still, from Figure 4, two groups could be distinguished: one formed by patients presenting none or mild effects and another composed of subjects who ranked moderate or severe side effects. Figure 4-B shows two box plots corresponding to these groups. As the median line of one box lies outside of the other one, there is likely to be a difference between the two groups. Spearman's test confirmed this aspect, which indicated a significant correlation between the symptoms and the cell survival rate, with a coefficient $r = 0.7909$ (p-value = 0.0003).

Smoking during and after radiotherapy can influence the quality of voice and hoarseness level after treatment [32], but all patients in this study said they had stopped smoking before the beginning of the treatment. All patients reported the levels of severity of dysphagia and odynophagia as very similar, and such difficulty in distinguishing those effects has already led other studies to characterize them as painful swallowing in general [33, 34].

Inflammation is a primary response to an injury and helps promote tissue repair, characterized by the sequential release of several mediators that regulate vascular permeability [35]. Considering this, patients with a higher survival rate of irradiated peripheral mononuclear blood cells also presented a higher degree of tissue inflammation, shown through side effects. Dysphagia and odynophagia are among the consequences of laryngeal edema, a side effect caused by radiotherapy. Edema occurs due to increased vascular permeability caused by blood and lymphatic vessel blockage and inflammatory mediators released from cells that have suffered radiation damage [36, 37].

Applying the methodology presented in this study could allow assessing genetic radiosensitivity factors to implement a patient-specific protocol. Some studies have estimated that, with individualized protocols, it is possible to increase by 20 % the success of treatment [38]. Severe adverse effects caused by RT in patients with head and neck cancer have been shown to decrease their quality of life, including deterioration in their relationship with family and friends [38, 39].

In this scenario, the existence of a test for predicting individual radiosensitivity, as a pre-radiotherapy step, could help the psychological preparedness of patients and evaluate strategies to optimize the treatment and improve patient quality of life. Thus, our findings motivate further investigation to establish a pretreatment test that evaluates irradiated peripheral blood mononuclear cell viability as a biomarker of individual radiosensitivity of patients undergoing laryngeal cancer radiotherapy.

4. CONCLUSION

For patients diagnosed with early-stage laryngeal cancer undergoing radiotherapy, this work indicates a positive correlation between the severity of dysphagia and odynophagia with the survival rate of in vitro irradiated peripheral blood mononuclear cells (PBMC), using the trypan blue exclusion test. On the other hand, no correlation was found between the viability rates of irradiated PBMC and the observed degrees of radiation dermatitis for the same subjects.

Our findings suggest that laryngeal cancer patients assigned to radiotherapy, presenting survival rates of in vitro irradiated peripheral mononuclear blood cells higher than 70% before treatment, will experience moderate to severe acute laryngeal side effects, for instance, dysphagia and odynophagia. Even though further studies in larger cohorts are needed to ensure this methodology's validity and reliability, this pilot study points to a practical and affordable pre-therapeutic test for predicting individual radiosensitivity.

5. ACKNOWLEDGMENTS

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - Brazil - Grant No: 308467/2015-9) and the International Atomic Energy Agency (IAEA-Austria - Research Contract No: 22266).

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