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Effect of Radiotherapy and Low-Level Laser Therapy on Circulating Blood Cells of Rats

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Abstract

Introduction: This study aimed to investigate the effect of low-level laser therapy (LLLT) on the blood cell count when applied to parotid glands of rats irradiated by volumetric modular arc therapy (VMAT).

Methods: Thirty-two adult male Wistar rats were used in this study. Samples were randomly assigned to three groups: control group (CG, n = 8), immediate laser group (24 hours) (ILG, n=12), and late laser group (120 hours) (LLG, n=12). The two laser groups were previously subjected to VMAT radiotherapy in a single dose of 12 Gy. LLLT with an AsGaAl laser (660 nm, 100 mW) was applied at three points in the region of the parotid glands, right side, with the energy of 2 J per point (20s, 70 J/cm^2) and a spot size of 0.0028 cm^2 for 10 consecutive days. In the euthanasia, blood samples were obtained by cardiac puncture. The samples from each group were processed by an automatic method and analyzed for erythrogram, leukogram and platelet count values. The data were analyzed by ANOVA and each LLLT time point was analyzed in relation to the control group, with a significance level less than 0.05.

Results: Groups using LLLT had higher red blood cell counts, being higher in the LLG ($p = 0.000$). The hematimetric indices MCV ($P=0.002$) and MCH ($P=0.009$) were lower than the control group, especially when compared to the group using LLLT 120h after radiotherapy (LLG). White blood cell counts were lower in the groups with radiotherapy and immediate use of LLLT (ILG) ($P=0.011$), mainly at the expense of lymphocytes ($P=0.002$).

Conclusion: The results suggest a potential systemic effect of LLLT, especially on circulating red blood cell counts, regardless of their time of immediate or late use of radiotherapy.

Keywords: Low-level light therapy; Radiotherapy; Blood cell count; Systemic effect; Rats.

Introduction

The use of low-level laser therapy (LLLT) in patients undergoing radiotherapy in the head and neck regions has shown favorable results both in the prevention and in the treatment of sequelae in the oral cavity, including mucositis and xerostomia caused by hyposalivation. Many studies in animal or clinical models have been developed with the objective of verifying the possible effects of LLLT on the repair of irradiated salivary glands, and their results have demonstrated positive effects in relation to cell proliferation, modulation of the inflammatory process, and induction of collagen synthesis accelerating the process of tissue repair.[1-4] In addition, it has been reported that the use of LLLT after radiotherapy increases the synthesis of proteins (aquaporins 1 and 5) that participate in the process of production and salivary secretion of the parotid glands.[5]

LLLT occurs by transferring energy to tissues without the use of heat and with limited radiation intensity. Such characteristics as wavelength, energy density and irradiated area have a strong relationship with their effect on tissues, which can stimulate or inhibit physiological and biochemical processes. The reactions resulting from LLLT are, among others, related to the increase of proliferative processes, vascular alterations and effects of biostimulation of the inflammatory process that contributes to facilitating and accelerating the tissue repair process.[6,7]

In addition to the local effects of LLLT, its possible systemic effect has been reported; however, experimental studies *in vitro* and *in vivo* have reported conflicting results.[8] In investigating the effect of LLLT on the distance microcirculation of rats,[9] no effect was observed, but in another similar study,[10] it was reported that the use

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of LLLT accelerated the angiogenesis and stimulated leukocyte chemotaxis. Furthermore, it was pointed out that when it was used in a single dose, it accelerated the inflammatory phase of tissue repair in the treatment of burns in rats.

In view of the reports, it is relevant to investigate the possible systemic effect of LLLT in order to clarify its action when used concomitantly with radiotherapy that has systemic effects, especially on circulating blood cells. The purpose of this study was to investigate the effect of LLLT on blood cell counts when applied to parotid glands of rats irradiated by volumetric modular arc therapy (VMAT).

Materials and Methods

This study is part of a larger study by Krueger et al,[5] and it was approved by the Ethics Committee on the Use of Animals (CEUA/ULBRA). Thirty-two adult male Wistar rats were used in this study. Samples were randomly assigned to three groups: control group (CG, n = 8), immediate laser group (24 hours) (ILG n=12), and late laser group (120 hours) (LLG, n=12). The two laser groups were previously subjected to VMAT radiotherapy. Radiotherapy by VMAT with linear accelerator Trilogy (RapidArc) (Varian, USA) in a single dose of 12 Gy, a beam of 6 MV photons, and an irradiation field of 9 × 40 cm^2 was applied to a 1cm circular area, delimited by a lead collimator in the cranial bone. The LLLT with Photon Laser III equipment (DMC, LTDA) was applied by a continuous laser source of AsGaAl with a wavelength of 660 nm on the right side parotid gland according to the study protocol by Krueger et al[5] (Figure 1). The total energy density/day was 70 J/cm^2 with a spot area of 0.0028 cm^2 and power of 100mW for 10 consecutive days. In the control group, all procedures were simulated (placebo).

Collection and Analysis of Blood Samples

The collection of blood samples from animals that had been fasting for three hours was performed at the time of euthanasia by cardiac puncture that occurred at the same time in the ILG and control group and five days later in the LLG (Figure 2). The 5 mL blood samples were distributed into standard collection tubes containing 10% sodium EDTA anticoagulant. The tubes were labeled separately according to the groups and stored under refrigeration at a temperature of 4°C. Afterwards, they were sent for analysis.

The samples were processed by the automated method, passing through the homogenization process (Fanem, model 270T) for 5 minutes. In the veterinary hematological analyzer Poch-100iV Diff (Roche), cell counts were performed to obtain the erythrogram composed of the erythrocyte or red blood cells (RBCs) count, hemoglobin (Hb), hematocrit (Ht) and hematimetric indices (mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], mean corpuscular hemoglobin concentration [MCHC] and red cell distribution range [RDW], and platelet (PLT) count. The leukogram was obtained by counting leukocytes or white blood cells (WBC) in smears stained with fast panoptic (Newprov) and observed under an optical microscope (Zeiss) with an immersion objective (×100 magnification) by a single

Figure 2. Cardiac Puncture for Blood Collection.

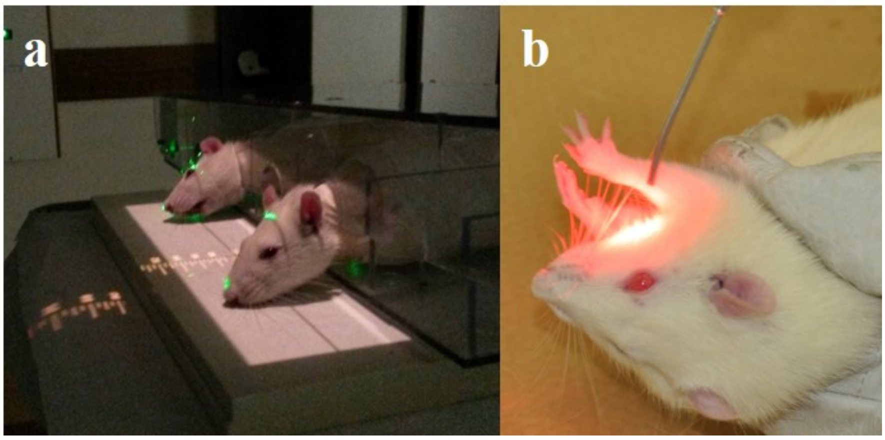

Figure 1. Rats Positioned to Radiotherapy (a) and Low-Level Laser Therapy Application Protocol (b).

researcher (veterinarian). We counted the absolute values of WBC and cell differentiation (segmented neutrophils, eosinophils, basophils, lymphocytes, and monocytes).

Statistical Analysis

In the SPSS program version 22.0 (San Diego, USA), the data were analyzed by the Shapiro-Wilks and ANOVA tests. The values of each time point of use of the LLLT were analyzed in relation to the control group, and $P<0.05$ was considered significant.

Results

For the hematological analysis, the blood count and platelet count values of all blood samples were considered (n=31). There was a loss in the ILG 48 hours after radiotherapy. The values obtained from the CG (Table 1) were used as a reference parameter for comparison with the ILG (Table 2) and LLG (Table 3) experimental groups. In the comparative analysis of the mean values between all groups, there was a significant difference in the increase in the erythrocyte count in the group with the late use of LLLT (LLG) ($P=0.000$). However, there was a significant reduction in the values of MCV ($P=0.002$) and MCH ($P=0.009$) in the experimental groups, with a greater reduction in the LLG. RDW values increased significantly in the experimental groups ($P=0.015$), with a greater increase in the LLG.

The values of Hb ($P=0.633$), Ht ($P=0.644$) and MCHC

Table 1. Distribution of Blood Cell Count Values in the Control Group (Placebo)

Hematological Variables	Mean	Median	Standard Error	Minimum Value	Maximum Value
RBC ($\times10^6$µL)	8.319	8.275	.3431	7.8	9
Hb (g/dL)	15.03	14.80	.861	14	17
Ht (%)	44.63	44	2.669	43	51
MCV (fL)	53.64	53.24	2.409	51	58
MCHC (g/dL)	33.68	33.79	1.002	32	35
MCH (pg)	18.06	18.22	0.739	17	19
RDW (%)	11.85	10.95	2.507	10	18
WBC (µL)	7612.5	7800	1711.672	5100	9800
Neutrophils (µL)	1311.25	1239	467.361	793	2240
Eosinophils (µL)	85.67	97	20.502	62	98
Lymphocytes (µL)	6249.13	6262	1535.983	3978	8232
Monocytes (µL)	80	80	25.456	62	98
PLT (µL)	1051250	1051000	108131.86	818000	1157000

Abbreviations: RBC (red blood cells); MCV (mean corpuscular volume); MCH (mean corpuscular hemoglobin); MCHC (mean corpuscular hemoglobin concentration); RDW (red cell distribution range); WBC (white blood cells); PLT (platelets).

Table 2. Distribution of Blood Cell Count Values in the Group With Immediate Use of Low-Level Laser Therapy

Hematological Variables	Mean	Median	Standard Error	Minimum Value	Maximum Value
RBC ($\times10^6$ µL)	8.767	8.700	.3279	8.3	9.3
Hb (g/dL)	15.68	15.80	.703	15	17
Ht (%)	43.56	44	1.944	40	46
MCV (fL)	50.59	51.10	3.164	46	56
MCHC (g/dL)	35.98	35.90	1.525	34	39
MCH (pg)	17.91	18	.888	17	19
RDW (%)	13.02	13.80	1.473	11	15
WBC (µL)	5070	4900	1609.727	3400	8400
Neutrophils (µL)	1282.10	1156.50	697.064	544	2538
Eosinophils (µL)	285	285	284.257	84	486
Lymphocytes (µL)	3721.8	3518	1321.275	2170	6132
Monocytes (µL)	41.50	41.50	7.778	36	47
PLT (µL)	996555.56	1017000.00	260722.60	531000	1422000

Abbreviations: RBC (red blood cells); MCV (mean corpuscular volume); MCH (mean corpuscular hemoglobin); MCHC (mean corpuscular hemoglobin concentration); RDW (red cell distribution range); WBC (white blood cells); PLT (platelets).

Table 3. Distribution of Blood Count and Platelet Count Values in the Group With Late Use of Low-Level Laser Therapy

Hematological Variables	Mean	Median	Standard Error	Minimum Value	Maximum Value
RBC (x10^6 µL)	9.250	9.350	.5108	7.9	10
Hb (g/dL)	15.17	15.45	2.104	9	18
Ht (%)	44.25	44	2.491	40	50
MCV (fL)	47.90	47.55	3.407	43	56
MCHC (g/dL)	34.47	35.15	4.699	20	39
MCH (pg)	16.35	16.70	1.737	11	18
RDW (%)	14.18	13.95	.867	13	16
WBC (µL)	7033.33	6700	1952.310	3500	10100
Neutrophils (µL)	2044.17	1761	1042.473	875	4141
Eosinophils (µL)	133.30	99.50	104.371	51	400
Lymphocytes (µL)	4871.08	4875	1293.776	2555	7311
Monocytes (µL)	51	51	--	51	51
PLT (µL)	1033500	1027000	127266.006	846000	1304000

Abbreviations: RBC (red blood cells); MCV (mean corpuscular volume); MCH (mean corpuscular hemoglobin); MCHC (mean corpuscular hemoglobin concentration); RDW (red cell distribution range); WBC (white blood cells); PLT (platelets).

($P = 0.333$) did not show significant differences between the groups. However, the mean values of the Hb and Ht dosages remained similar in the three groups.

The leukogram showed differences in the mean values of total leukocytes (WBC) ($P = 0.011$) with a greater decrease in group ILG with the immediate use of LLLT. In the cellular differentiation of WBC, the mean values of lymphocytes showed a significant decrease (lymphocytopenia) in the experimental groups ($P = 0.002$), mainly in the ILG. The differences in values of segmented neutrophils (p = 0.064), eosinophils ($P = 0.223$) and monocytes ($P = 0.313$) did not show significant differences in relation to the control (CG). However, eosinophilia was observed in both experimental groups, with a greater increase in eosinophils in the ILG and monocytopenia in the same group (ILG). The counts of segmented neutrophils were shown to be lower (neutropenia) in the ILG and increased (neutrocytosis) in the LLG. Basophils were not observed in any of the groups.

The platelet (PLT) count showed a decrease in the mean values in the two experimental groups when compared to the CG; however, that difference was not statistically significant ($P = 0.238$).

Discussion

The local effects of LLLT, when used in salivary glands after radiotherapy of the head and neck regions, are well described in the literature.[1,4,5] However, few studies on its effects on adjacent areas or its systemic effect have been investigated, mainly in relation to circulating blood cell counts, which, to date, has not been reported in the literature.

Thus, the hematological analysis from the reference values obtained from the control group allowed identifying, in the groups using LLLT, the increase in the red blood cell counts, mainly when the LLLT was used late or 120h after radiotherapy. In addition, it was possible to identify significant differences in some hematimetric indices of the experimental groups, which may contribute to the hypothesis of a possible systemic effect of LLLT.

Even though it is known that the infrared laser has greater power to penetrate tissues than the red laser,[8] in the present study, a red laser (660 nm, power 100 mW) was used with an energy density of 2 J/cm^2 per point, distributed in three different points, which promoted a better distribution of the laser energy in the region of its application and a possible effect on the blood cell count.

Among the hematimetric indices, the experimental groups showed a significant decrease in the MCV, regardless of the immediate or late use of LLLT. However, this can be directly related to the effects of radiotherapy[11,12] and/or to the effect of LLLT.[13,14] In the study by Al Musawi et al,[14] in blood samples *in vitro*, the MCV decreased significantly. We emphasize that the radiation dose of 72 J/cm^2 used in the study by Al Musawi et al was the factor associated with this decrease in MCV. Thus, with the exposure of erythrocytes to LLLT with a higher total irradiation dose and with a higher penetration wavelength as used in the present study, the hypothesis that LLLT may cause a reduction in the volume of erythrocytes is created.

This is due to the fact that LLLT is able to break the cell membrane, leading to an increase in the porosity and attraction of the calcium ions that are free in the extracellular solution (EDTA solution) and migrate to the intracellular fluid of the erythrocytes. Thus, this increase induces the flow of K + ions into the extracellular fluid, which leads to a decrease in the MCV of erythrocytes.

Both the significant decrease in the MCH and the increase in the red cell distribution range in the blood (RDW) are directly related to the decrease in MCV in the experimental groups. On the other hand, the mean corpuscular concentration in each erythrocyte (MCHC) remained similar in all groups, as well as the total dosages of Hb and Ht, which may suggest a beneficial effect of LLLT on the levels of Hb and Ht,[15,16] after the previous radiotherapy that has a deleterious effect on blood cells.[12,17,18] This result may be related to the photobiostimulator effect of the laser at a wavelength of 660 nm, which can cause a reaction with Hb since it has chromophoric receptors that are activated in the presence of light.[13,19]

This result creates the hypothesis of a possible systemic effect of LLLT because with the increase of erythrocytes, particularly Hb, the transport of oxygen to the tissues increases and can contribute to the repair and healing process. Clinically, this effect could be interpreted by the acceleration of the tissue repair and healing process, as there will be a greater blood supply through vasodilation (hyperemia) and consequently an increase in tissue oxygenation, which contributes to the acceleration of the tissue repair process.

White blood cell (WBC) counts in the experimental groups had a significant reduction (leukopenia). During radiotherapy, a progressive decline in the total leukocyte count tends to occur in the 1st week of treatment, with a marked decrease occurring up to the 4th week.[20] Therefore, the decreased count of WBC verified, especially in the group using LLLT immediately after radiotherapy (ILG), may be associated with this deleterious action on white blood cells.[21]

However, the group with the longest period of time between radiotherapy and euthanasia (LLG) showed a leukocyte count close to the control group. This time interval may have contributed to the recovery of bone marrow and consequently to the production of white blood cells.[22] However, LLLT may also have contributed to cell recovery. As reported in another study,[23] this increase in leukocytes was associated with an increase in mitochondrial intracellular ATP resulting from laser radiation when used at a wavelength of 589 nm and fluency of 72 J/cm^2, a parameter closes to that used in the present study.

On the other hand, we must also consider the possible limitations of the present study. Among them, the choice of the animal model (rats) may present confounding factors related to the time/age and metabolism of the animals, which makes comparison with humans difficult.[24] In addition, hematological parameters in rodents may vary according to different strains and strains of a species[25]. Variations related to sex, age, handling, environment, circadian rhythm and the method used in blood collection can generate conflicting results.[26-29]

However, the use of a control group, besides serving as a parameter in the identification of small individual variations in the variables investigated in the experimental groups, made it possible to minimize possible biases. The use of simulated procedures (placebo) in the control group aimed to maintain the handling of animals with the same parameters and level of stress (the placebo effect) caused in the other groups. Another positive point was the analyses carried out by a single researcher (veterinarian) who reevaluated the samples in an interval of seven days, which aimed to verify the reproducibility of the results of the hematological analyses.

The results obtained in the present study may contribute to the hypothesis that LLLT has a positive effect on maintenance or even an increase in red blood cell counts responsible for tissue oxygenation, even after the deleterious effects of radiotherapy. Therefore, studies with greater power of evidence should be conducted to confirm this hypothesis.

Conclusion

The results of the hematological analysis suggest a potential systemic effect of LLLT on circulating blood cell counts, especially in red blood cells, regardless of its immediate or late use with VMAT radiotherapy. However, leukopenia at the expense of lymphocytes was compatible with the effect of radiotherapy on circulating blood cells, with no immediate effect of LLLT on white blood cells.

Conflict of Interests

The authors declare that they have no conflict of interest.

Ethical Considerations

This study has been approved by the Animal Care and Use Committee of Universidade Luterana do Brasil – RS/Brazil (CEUA/ULBRA; approval number 2014-10P).

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