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Research article

Changes in splenic uptake pattern associated with X-ray irradiation

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ABSTRACT

Purpose: To evaluate the splenic uptake function after irradiation with high-energy X-rays.
Materials and methods: Fourteen male Wistar rats were distributed into three groups. Group 1 (n = 6) – control, non-irradiated; Group 2 (n = 4) – animals that were irradiated and studied 24 h after irradiation; and Group 3 (n = 4) – animals that were irradiated and studied 48 h after irradiation. The animals were irradiated with 8 Gy X-rays in the abdominal region. According with the groups, after 24 or 48 h, 1 ml/kg of a 50% colloidal carbon solution was injected in the left internal jugular vein. After 40 min, the spleens were removed for histological studies. Macrophages containing carbon pigments in their cytoplasms were counted in 16 consecutive microscopic fields, and their means were considered as the uptake pattern of each animal.
Results: In the control groups, carbon pigments were captured by macrophages in the red and white pulps, while in the irradiated groups, the uptake in the marginal zone, around the white pulp, was enhanced. There was no disorder on the splenic parenchyma or necrosis in histological analyzes. Qualitatively rare apoptotic events were observed, with no difference between control and irradiated animals.
Conclusion: The high-energy X-ray, used in radiotherapy, modifies the splenic clearance, enhancing the amount of marginal zone macrophages containing colloid particles. This radiation was not associated with morphological changes, nor with necrosis or apoptosis of splenic tissue.

1. Introduction

The spleen is the largest lymphoid organ, serving the function of the body's defense in removing foreign particles from the blood, as well as playing a pivotal role in the bilirubin, lipids, and amino acids metabolism [1]. This organ also controls the circulating leukocytes and platelets, as well as the maturation of the lymphocytes, among other activities, such as the removal of old red blood cells and the storage of metals, especially iron from the metabolism of hemoglobin [1, 2, 3].

Splenic macrophages are located around the sinusoidal capillaries, forming the Billroth's splenic cords in the white and red pulp, as well as in the marginal zone, around the white pulp. Since the beginning of 1990, the splenic uptake activity has been investigated regarding alcoholism [4], from both partial splenectomies and autogenous splenic implants [5, 6, 7, 8, 9, 10, 11, 12], as well as from oophorectomy and pregnancy [13] in its activities.

Radiotherapy (RT), commonly used in cancer as effective anti-tumor and pro-immune modulator, induces the openings of DNA molecules, which leads to cell death by apoptosis or necrosis [14]. RT is an immunosuppressant [15], provokes lymphopenia [16, 17], and forces macrophages to release cytokines, which are modulated by B and T lymphocytes [18].

RT modifies the tumor microenvironment, damaging blood vessels and peritumor cells of the immune system. The aggression to endothelial cells caused by RT induces to an intense inflammatory process with injuries and dysfunctions in all local tissues. Disorders on vessels inhibit the infiltration of CD8 + lymphocytes into tumors, and activate immunosuppressive pathways that lead to the accumulation of radioresistant suppressor cells, such as M2 macrophages and regulatory T cells. These events are associated with decreasing in the effectiveness of RT in destroying the tumor [19].

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Primary splenic tumors are rare and, in general, resistant to RT [20]. However, the spleen can suffer the effect of RT even when it is outside of the radiation field [21], as it was proved in the management of lower esophageal and gastric cancers treatment, which also distresses the splenic function [22].

Patients with follicular lymphoma restricted to the spleen may be treated with RT with curative intent, and approximately half of these patients recover with radiation alone [23].

Post-irradiation effects on the spleen size have been evaluated by computerized tomography scan in ninety gastric cancer patients treated with 45 Gy in 25 fractions. The authors observed a progressive radiation dose-dependent reduction of spleen volume as well as an increasing in the occurrence of pneumonia and fatal sepsis, probably related to the hyposplenia [22].

Due to the possible toxic effect of the radiation to the spleen, this study sought to investigate the influence of RT upon the splenic uptake function.

2. Material and methods

This method was approved by the Committee on Animal Research and Ethics from the Federal University of Minas Gerais (UFMG) (logged under CEUA-Protocol:115/2018), and carried out according to the provisions of the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Fourteen eight-week male Wistar rats with an average weight of 265 ± 17 g, and placed in the Biotherium of the UFMG School of Medicine, were studied. The animals were placed in appropriate cages, received food and water ad libitum, and were distributed into the following three groups:

Group 1 (n = 6) – non-irradiated animals (control group). After four hours fasting, and under intraperitoneal anesthesia, consisting of ketamine hydrochloride (80 mg/kg), associated with xylazine hydrochloride (7 mg/kg) [24], the animals were submitted to 1 ml/kg of a 50% colloidal carbon solution injection in the left internal jugular vein. After forty minutes, they were euthanized with 240 mg/kg of ketamine hydrochloride, associated with 21 mg/kg of xylazine hydrochloride [24]. The spleen was removed and immersed in a 10% buffered formaldehyde solution for histological study using 4 μm splenic slices set on glass slides, following the habitual pathological technique and stained with hematoxylin and eosin.

Group 2 (n = 4) – animals irradiated with 8 Gy (4 Gy in anteroposterior and 4 Gy in posteroanterior incidences) in the abdominal region, using an isocentric technique [25] by means of a 6 MV linear accelerator at the Radiotherapy Center of the Luxemburgo Hospital of Belo Horizonte, Brazil. Twenty four hours post-irradiation, the rats were submitted to 1 ml/kg of a 50% colloidal carbon solution injection in the left internal jugular vein and then studied according to the protocol described in Group 1.

Group 3 (n = 4) – animals irradiated with 8 Gy (4 Gy in anteroposterior and 4 Gy in posteroanterior incidences) in the abdominal region, using an isocentric technique [25] by means of a 6 MV linear accelerator. Forty eight hours post-irradiation, the rats were submitted to 1 ml/kg of a 50% colloidal carbon solution injection in the left internal jugular vein and then studied according to the protocol described in Group 1.

The number of animals used in this study was in accordance with the recommendations of the ethics committee. The number of animals was calculated using the Scheibe method [26], considering a level of significance of 5% for p-value, coefficient of variation between 15% and 20%, and an expected difference of up to 20% between irradiated and control groups. There was the possibility to perform all statistical calculations with consistent significance.

The 8 Gy dose used in this work was based on previous studies conducted in rats [27, 28, 29]. Other doses of radiation and even fractionated schemes may be probably adequate and applicable in the treatment of specific tumors. However, the option for an established dose for the irradiation of the animals was necessary to achieve reliable results.

The observation time of 24 h and 48 h was chosen to verify the influence of irradiation on the splenic tissue during short and variable periods. The greatest damage due to RT occurs during this period, after which complications occur mainly as result of inflammatory response, which is more significant after the second post-operative day.

The macrophages containing colloidal carbon pigments in their cytoplasm were counted in 16 consecutive optical microscopic fields on each slide, using a magnification of 1000X, in immersion oil. The presence of these macrophages was identified in the different regions of the spleen, using an optical microscope at a magnification of 200X, and its quantity was estimated using the Image J software [30], which made direct calculations in the splenic parenchyma, divided by areas.

The mean numbers of the macrophages containing colloidal carbon pigments in the 16 fields were compared among the groups, two by two, by the two-tailed Student t test. The mean of the area fractions in the splenic marginal zones containing colloidal carbon were assessed and compared among the groups, two by two, by the one-tailed Student t test. Differences corresponding to $p < 0.05$ were considered significant.

3. Results

The follow-up of the animals during the experiment was uneventful. No difference was observed among the averages of macrophages that encompass colloidal carbon among the studied groups (Table 1). In the control group, the macrophages containing colloidal carbon were distributed in both the red pulp and the marginal zone. However, in the two groups submitted to irradiation, the macrophages containing colloidal carbon were located almost exclusively in the marginal zone (Tables 2 and 3). The Figure 1 presents the colloidal carbon distribution associated with the RT effect. The spleen of the rats submitted to RT presents the distribution of the colloidal carbon mainly surrounding the white pulp, which is the marginal zone (Figure 1).

There was no disorder on the splenic parenchyma or necrosis in histological analyzes. In the pilot study of this investigation, qualitatively rare apoptotic events were observed using the Tunel technique, with no difference between control and irradiated animals. For this reason, apoptosis was not studied in this work.

4. Discussion

The literature has established that RT in high doses and multiple applications causes harm to the splenic lymphoid tissue, such as hypotrophy, due to damage to the white pulp [20]. In this study, no macro- or microscopic morphological abnormality was detected in the spleen parenchyma. It is important to consider that the period of observation of the splenic tissue was short and may not have been enough to show possible effects of the X-rays on the splenic structure.

Table 1. Mean values (M) and standard error of the mean (SEM) of the quantity of macrophages containing colloidal carbon pigments per group of animals.

Groups	N	M ± SEM
1	6	429.2 ± 35.9
2	4	410.3 ± 9.6
3	4	432.2 ± 7.6

N: Total number of animals.
Group 1: Control – non-irradiated animals.
Group 2: Animals irradiated with 8Gy in the abdomen and euthanized 24 h after irradiation, 40 min after intravenous injection of colloidal carbon solution.
Group 3: Animals irradiated with 8 Gy in the abdomen and euthanized 48 h after irradiation, 40 min after intravenous injection of colloidal carbon solution.
Comparison among groups: $p > 0.05$.

Table 2. Mean values (M) and standard error of the mean (SEM) of the area fraction around the white pulps occupied by carbon.

White pulp	N	M ± SEM
Group 1	13	0.01191 ± 0.0016 *
Group 2	8	0.02885 ± 0.0010 **
Group 3	7	0.03664 ± 0.0031 ***

N: Number of white pulps.
Group 1: Control – non-irradiated animals.
Group 2: Animals irradiated with 8 Gy in the abdomen and euthanized 24 h after irradiation, 40 min after intravenous injection of colloidal carbon solution.
Group 3: Animals irradiated with 8 Gy in the abdomen and euthanized 48 h after irradiation, 40 min after intravenous injection of colloidal carbon solution.
Comparison among: * and **, $p < 0.0001$; * and ***, $p < 0.0001$; ** and ***, $p = 0.023$.

Table 3. Mean values (M) and standard error of the mean (SEM) of the quantity of macrophages in the red pulp containing colloidal carbon pigments per group of animals.

Groups	N	M ± SEM
1	6	201.7 ± 15.2 *
2	4	35 ± 1.3 **
3	4	42 ± 2.1 ***

N: Total number of animals.
Group 1: Control – non-irradiated animals.
Group 2: Animals irradiated with 8Gy in the abdomen and euthanized 24 h after irradiation, 40 min after intravenous injection of colloidal carbon solution.
Group 3: Animals irradiated with 8 Gy in the abdomen and euthanized 48 h after irradiation, 40 min after intravenous injection of colloidal carbon solution.
Comparison among: * and **, $p = 0.0014$; * and ***, $p = 0.0048$; ** and ***, $p = 0.45$.

In the non-irradiated animals, the pigments of colloidal carbon were removed by both red pulp and marginal zone macrophages. On the other hand, the location of the splenic macrophages that uptake the colloidal carbon was dramatically modified by the radiation. After being submitted to X-rays, either after 24 h or 48 h, the clearance of the colloid carbon was performed almost exclusively by the macrophages of the marginal zones that surround the white pulps. The macrophages from the rest of the splenic parenchyma had irrelevant participation in this colloid removal. This result is in accordance with previous studies in mice submitted to low-energy X-ray irradiation of the entire body, which showed that phagocytosis of *Shigella* was also impaired by irradiation [31].

The irradiation of the spleen is associated with less uptake of colloid carbon particles by the red pulp macrophages without apoptosis, necrosis or any detectable macro or microscopic morphological alteration. Therefore, the absence of colloidal carbon uptake in most of the splenic parenchyma may be not due to the absence of macrophages in the red pulp, but to the loss of their functional capacity. Another possibility is the change in the vessels functionality influencing the colloidal carbon drainage, not only from blood stream, but also from the lymphatic system. On the other hand, radiation did not interfere with the ability of macrophages in the marginal zone to remove colloid pigments. Previous studies have shown that radiotherapy induces changes in macrophages, which increase their uptake capacity and modulate their phenotype to become pro-inflammatory [32]. Cytogenetic and molecular studies may contribute to understanding the mechanism associated to these observations.

Our previous studies on blood clearance of bacteria and colloidal carbon conducted in this line of research described the blood clearance of *E. coli* and colloidal carbon preserved even after the trauma, including large surgical procedures on the spleen in animals and human beings [5, 6, 7, 8, 9, 10, 11, 12, 13], as well as many of our own references in animals and humans [33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44]. However, this work proved that a single X-ray irradiation may influence the splenic blood and lymphatic flow clearance more than any spleen mechanical trauma and surgeries, even during a long period.

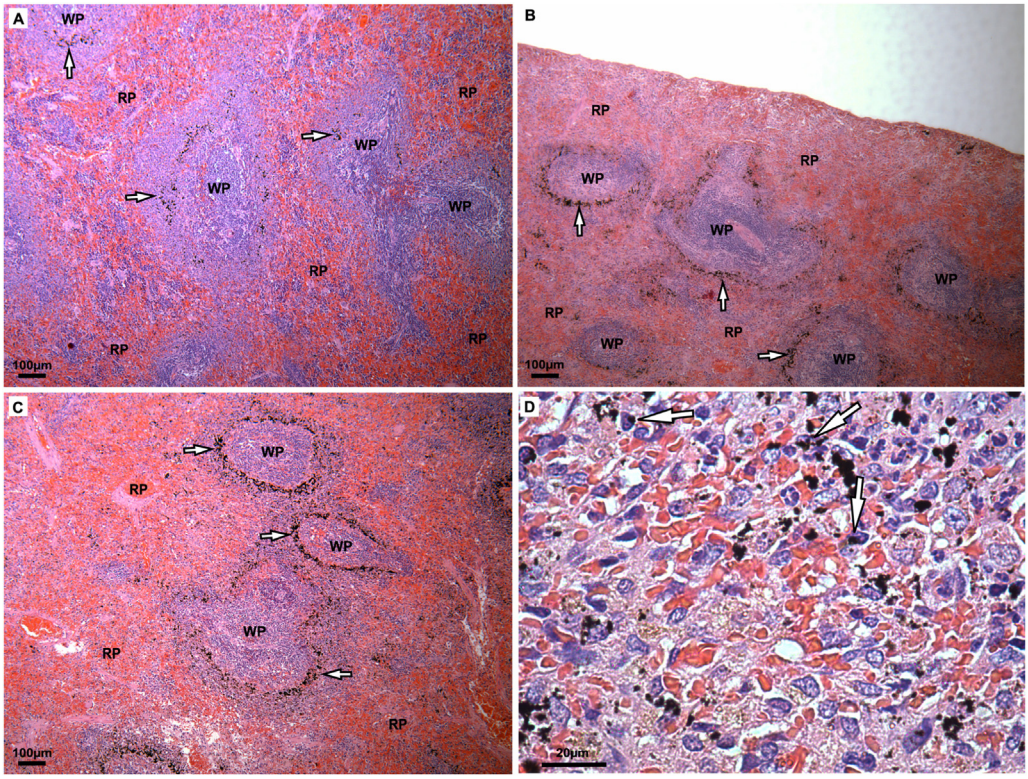

Figure 1. Distribution of the colloidal carbon pigments in the splenic tissue. A – Distribution of the macrophages containing colloidal carbon (arrows) in the non-irradiated spleens (Group 1) (H&E, 100 X). B – Concentration of the macrophages containing colloidal carbon almost exclusively in the marginal zone around the white pulps (arrows) in the spleens studied 24 h after irradiation (Group 2) (H&E, 100 X). C – Concentration of the macrophages containing colloidal carbon almost exclusively in the marginal zone around the white pulp (arrows) in the spleens studied 48 h after irradiation (Group 3) (H&E, 100 X). D – Examples of macrophages containing colloidal carbon pigments in their cytoplasms (arrows), in the splenic tissue of the non-irradiated animals (Group 1) (H&E, 1000 X).

The clearance by the macrophages of the splenic marginal zone is of great relevance and brings additional information to the knowledge about RT, since the literature mentions a possible reduction in the spleen function after RT, demonstrated by gene expression and proteins involved in the signaling cascade for apoptosis [22, 45, 46].

The lymphoma of the splenic marginal zone is quite rare and is characterized by changes in B lymphocytes, caused by a disruption of genes in cell differentiation. The marginal zone, located around the white pulp, is the location of the entrance and presence of circulating B and T lymphocytes, macrophages, immature dendritic cells, and fibroblasts [47]. The marginal zone of rats and mice, but not humans, contains a type of macrophage called the metallophilic, which acts in the early stages of immunity [48].

No consensus has been reached concerning the treatment of a marginal zone lymphoma, which is based on monotherapy with rituximab, an anti-CD 20 monoclonal antibody. Splenectomy may be indicated only for patients who are resistant to chemotherapy, but the results are uncertain [49, 50]. RT with a single dose of 6 Gy–8 Gy was applied in marginal zone lymphomas when villous lymphocytes were found in the peripheral blood, with transitory response for no more than one year in three of seven treated patients [51].

The resistance of the marginal zone to RT, as found in our study, may well be responsible for the lymphoma's recurrence. This work showed that the marginal zone is resistant to RT. As there are not previous works in the literature concerning the marginal zone radioresistance, further studies such as cytogenetic experiments may be necessary to understand this region of the spleen and its pathophysiology.

5. Conclusion

The high-energy X-ray, used in radiotherapy, reduces the uptake function of the red pulp macrophages what may be associated to the loss of their functional capacity as well as to alterations in splenic blood flow and drainage. However, the macrophages of the marginal zone preserve their capacity to remove colloid particles. An enhancement of their uptake function or even changes in the spleen vessels functionality may well be responsible for such behavior. Further investigations may be necessary to clarify the mechanisms.

This radiation was not associated with apparent morphological changes, nor with necrosis or apoptosis of splenic tissue. The observations indicate radioresistance of the marginal zone 24 h and 48 h after irradiation.

Declarations

Author contribution statement

Fernando P. de Faria: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Andy Petroianu: Conceived and designed the experiments; Wrote the paper.

Paula P. Campos, Marcela G. T. de Lazari: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Jony M. Geraldo, Clara B. Nascimento: Performed the experiments.

Sávio L. Siqueira: Conceived and designed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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