



Inhibition of nitric oxide synthase activity and chemokine (CXCL12) supplementation can improve hematopoietic reconstitution in mice lethally irradiated by ^{60}Co gamma radiation

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ABSTRACT

Reduction of nitric oxide (NO) production is related to increased survival in some models of infection and ionizing radiation (IR) exposure. The work used lethally irradiated (^{60}Co , 8Gy) C57Bl6j mice, treated or not with aminoguanidine (AG), an inhibitor of an isoform of nitric oxide synthase (iNOS). Also tested iNOS^{-/-} knockout mice and a distinct group treated intraperitoneally with synthetic CXCL12, a homing chemokine related to hematopoietic reconstitution after IR exposures. Aminoguanidine treatment lead to an overshoot of proliferation of hematopoietic CD34⁺ cells in bone marrows (day 2 after IR) and spleens (days 2 and 4 after IR) of irradiated mice, showing a compensative response of these organs against deleterious effects of radiation. CXCL12 mRNA production was increased on spleens of AG-treated mice at day 2 after IR, but not on other periods neither in bone marrows. CXCL12 administration did not alter CD34⁺ counts but seemed to keep circulating platelet counts in levels comparable to controls. Thus, CXCL12 and AG administration could help on bone marrow repopulation after critically exposed individuals.

Keywords: nitric oxide, CXCL12, hematopoiesis

1. INTRODUCTION

Exposure to ionizing radiation (IR) can induce clastogenic effects in biological systems. In unicellular organisms, these effects are clearer and more evident due to its reduced complexity [1]. On the other hand, IR-exposed multicellular organisms can develop a wide range of symptoms, dose-dependent or not. In humans, total or partial body exposures induce well characterized syndromes. Even in low dose/dose rates incidences, IR-exposed individuals can suffer from radiation-induced hematopoietic syndrome (RIHS), characterized by early neutropenia and thrombocytopenia and late erythrocytopenia [2]. In this way, an IR-exposed person would require urgent and sometimes expensive clinical care regarding its immunological safety and spontaneous bleeding and anaemia prevention. In some cases, bone-marrow transplantation (BMT), if available, can be prescribed and constitutes the ultimate and technically more efficient solution for hematologic recovery from IR exposures [3].

Pancytopenia is also frequent in exposed individuals, and it is related to disseminated death of hematopoietic cell precursors. Their increased radiosensitivity due its increased proliferation potential is an important inducing RIHS factor, and studies on radioprotection of bone marrow cells were carried to assess which therapy could be employed to better recover these patients [4–6]. These studies tested some important parameters, as repopulating potential of hematopoietic stem cells using CFU (colony-forming unit) assays, and cell-surface phenotyping by flow cytometry techniques to determinewhich cell populations can potentially rescue from IR-induced effects, and which can keep their proliferation and differentiation capabilities. Its expression was observed in hematopoietic precursors [7] and in endothelial cells [8]. In a simplistic manner, counts of cells bearing a CD34⁺ phenotype frequency in hematopoietic organs cell suspensions can be a start point to determine whether a therapy may be effective in recovering the cellularity of hematopoietic tissues Many protocols relying in CD34+ counts were described for this purpose, including clinical uses [9, 10].

To the moment, cytokine-based cocktails, mostly constituted by G-SCF (Granulocyte colony-stimulation factor), and SCF (Stem cell factor) [11] showed the most promising results. Although its prominent efficacy, cytokine-based therapies have elevated costs because of the need of proteins with high levels of purity and activity. A primordial chemokine, *CXCL12* (stromal cell-derived factor CXC motif chemokine ligand factor 12) was included in these cocktails after the studies re-

ported by some authors. The death and activation processes of stem cells are controlled by a group of mesenchymal cells, with various denominations, which are sensitive to tissue destruction and, upon termination of the offending event, are able to promote activation of those stem cells and recovery of hematopoietic tissue [12]. These mesenchymal populations occur in low prevalence in tissues, usually serving as support for many effector cells. Their function relies on intercellular messaging to attract or activate target cells in environmental balance and in contact with many other cell populations [13]. One important product of these cells is *CXCL12* (Stromal cell-derived factor), name of a group of CXC chemokines that are expressed into six isoforms, being α and β the most important ones [14], derived from different transcripts of a single gene, SDF-1, located on chromosome 10. The action of *CXCL12* is related to the presence of its specific receptor, CXCR4, which when activated can promote the proper settlement of primary mesenchymal cells [15]. The importance given to this chemokine has increased due to evidence that its action can improve homing of stem cells in bone marrow after transplantation [16]. Using another approach to study the hematopoietic recovery phenomena after irradiation, some molecules were already described as modifiers of response to radiation, which can minimize IR deleterious effects. The most common mechanism relies on the radical scavenging by vegetal flavonoids [17, 18], for example.

Nitric oxide is produced by mammalian cells mainly through L-arginine degradation into L-citrulline and NO by a family of nitric oxide synthases, named NOS. These synthases are described as having three main isoforms: nNOS, neuronal, eNOS (endothelial) and iNOS, formerly named as the inducible form of NOS [19].

iNOS (Nitric oxide synthase - inducible) was described as an important factor in immune responses against infectious agents [20, 21], and its importance in radiation induced damage has been studied in last years. Increased amounts of NO can be found in tissue sites damaged by IR effects, by iNOS over expression of damaged cells or by macrophages [22], and NO can react with peroxides that are also present in living cells after IR exposures, producing peroxynitrite (ONOO^-) and inducing more DNA damage than originally observed soon after exposure [23, 24].

Reduction of iNOS expression or its activity was proposed by some authors as one possible protection pathway against IR induced damage in exposed cells. Among described inhibitors, one of the most studied is aminoguanidine [25]. The release of NO by irradiated cells is related to apop-

tosis triggering through p53 activation [26]. Its production during inflammation is performed by iNOS, after events initiated by NF- κ B activation [27], through oxidization of L-arginine and induced by inflammatory cytokines such as IL-1 β (Interleukin 1-beta) and TNF- α (Tumour necrosis factor - alpha) [22] and IFN- γ (Interferon - gamma) [28].

This work aimed to test whether orally administered aminoguanidine or iNOS genetic knockout could affect haematological parameters as platelet counts in blood samples collected from gamma-irradiated animals by a ^{60}Co source in a single 8Gy dose and compare its effects with administration of a homing chemokine, *CXCL12*. This study also tested if aminoguanidine and/or *CXCL12* administration can reduce tissue ablation or increase counts of progenitor CD34 $^{+}$ cells at hematopoietic sites. Additionally, *CXCL12* mRNA expression was tested in 8Gy irradiated mice trying to relate chemokine expression levels, iNOS inhibition and hematopoietic damage after IR exposure.

2. MATERIALS AND METHODS

2.1. Aminoguanidine administration

Aminoguanidine (Sigma A-7259) was dissolved in water upon H₂SO₄ titrating in a 2g/L concentration. Solution had their pH corrected to 7.0 and were given to treated group mice as drinking water during all experiment. It was assumed that aminoguanidine hemisulphate was formed after titration and therefore administrated orally to animals as drinking water *ad libitum*.

2.2. Synthetic mouse CXCL12 administration

Liophilized synthetic mCXCL12 was a kind gift from Prof. Nobutaka Fujii (Kyoto University) and was diluted in sterile PBS. CXCL12 treated group received 1 μ g/kg intraperitoneally at days 2, 4 and 8 after irradiation.

2.3. Mice

Mice were raised on the colony of the Faculty of Medicine of the University of São Paulo and kept in adequate conditions according to Experimental Animal Use Committee of Tropical Medicine of the University of São Paulo and Animal Ethics Commission of IPEN. Groups of male six-week old C57Bl/6j mice (n=5) obtained from Medicine School Animal Facility (Sao Paulo University) and maintained at Protozoology Laboratory of Institute of Tropical Medicine of Sao Paulo. iNOS^{-/-} knockout mice were obtained from animal facilities of Biomedical Sciences Institute (University of Sao Paulo). All groups were gamma-irradiated inside a ⁶⁰Co panoramic source (Yoshizawa Kiko Co.) at the Center of Radiation Technology (IPEN/CNEN – SP) with a single 8 Gy dose, at 70cm from source and behind a 70% attenuator (total attenuated dose rate: 3.372kGy/h). Animals were immobilized in ventilated PVC capsules (10cm length, 2cm diameter) during irradiation events to prevent from heterogeneous exposure due to movement of mice during the event. Mice were sacrificed by CO₂ inhalation.

2.4. Experimental groups

Mice were classified according with their genotypes or received treatments: nt (non-treated, irradiated group); AG (aminoguanidine-treated, irradiated group); iNOS^{-/-} (iNOS knockout mice, irradiated group); CXCL12 (1µg/kg intraperitoneally at days 2, 4 and 8 after irradiation of synthetic mouse CXCL12, fed with tap water); CXCL12+AG (same as last, but have received aminoguanidine 2g/L in drinking water), NIC – CXCL12 (non-irradiated controls, treated with CXCL12 as above), NIC – AG (non-irradiated controls, but received aminoguanidine 2g/L in drinking water).

2.5. Blood sampling

Blood samples were collected from mice (n=5) at days 0 (before irradiation), 2, 4 and 8 after irradiation. After cutting tail ends, 2µL samples of total blood were collected with pipette tips appropriately treated (immersion for 10 minutes) in sterile citrate-dextrose anticoagulant solution [0.1M Na₃C₆H₅O₇, 0.11M C₆H₁₂O₆, 71mM C₆H₈O₇] in deionized water]. Aliquots received an equal volume of turkey erythrocyte suspension (genus *Meleagris*) fixed in buffered formaldehyde and diluted

in foetal bovine serum to concentration of 3×10^7 erythrocytes/mL. The concentration of mouse red cells and platelets was then determined by the ratio between counts and visualized turkey cells in 25 sight fields. Smears covered surface of glass slides and cells were stained soon after air drying using Leishman's staining.

2.6. Histological analysis

Mice were sacrificed at days 2, 4 and 8 after irradiation procedures. Spleen and femur fragments were aseptically removed and kept 24 hours in Sorensen's buffered formaldehyde at 4°C before paraffin embedding. Slices were placed on glass slides, treated and stained by hematoxylin-eosin method, visualized at 10 and 40 X magnifications and screened for visual confirmation of tissue destruction. Only samples from nt, AG and iNOS^{-/-} groups were studied by this method.

2.7. Semi-quantitative RT-PCR to CXCL12 mRNA quantification

Two individuals from 8Gy non-treated, aminoguanidine-treated and iNOS^{-/-} groups were tested for mRNA *CXCL12* chemokine quantification using a semi-quantitative RT-PCR approach. For that, 100mg (spleens) or 10^6 cells (bone marrows) were collected from mice irradiated at 8Gy, stored in TriZOL® reagent (Invitrogen) and processed following manufacturer instructions. After extraction, the RNA solutions were quantified by UV spectrophotometry, its concentration adjusted to 5µg/µL and used as template for the construction of the corresponding cDNA libraries using Oligo-dT₁₂₋₁₈ primers in reactions with presence of M-MuLV reverse transcriptase. Obtained cDNA libraries were used as template for amplifications of a fragment of 410bp belonging to the *sdf-1* gene, which encodes murine *CXCL12* gene (GenBank: NM_021704, 1837 bp) by PCR reactions using specific buffer (7.5mM Tris-HCl, pH 9.0. 0.2mM MgCl₂, 5mMKCl, 2mM (NH₄)₂SO₄), dNTP's (2.5 µM) and Taq DNA polymerase (5U/µL). The specific primers for amplification of *CXCL12* (410bp) from cDNA templates were: CTCGGTGTCTCTTGCTGTCCA (Fsdf-1) and GGCCCTCCTCCCCACCACT (Rsdf-1), 1.5µM each. The reactions were carried for 35 cycles with annealing temperature at 72.2 °C. As amplification controls, amplified products from murine β-actin were used. Mouse β-actin products were amplified using TGG AATCCTGTGGCATC-

CATGAAAC (F) and TAAAACGCAGCACAGTAACAGTCCG (R) primers, also for 35 cycles and annealing temperature to 55.6 °C (349 bp) [29]. The products of these amplifications were resolved in polyacrylamide gels (6%) in TBE (Tris-Borate EDTA, pH = 8.0), stained with silver nitrate impregnation and oxidization by formaldehyde and NaOH. Gels were photographed, and band optical densities were digitally obtained by measuring the intensity of staining of individual bands by ImageJ software (v.1.36). - The quantification of each band that represents the mRNA expression of *CXCL12* was given as the relative optical density units in relationship to optical densities measurements of β -actin bands, for each sample. Only samples from nt, AG and iNOS^{-/-} groups were studied by this method.

2.8. Determination of CD34⁺ counts by flow cytometry

Spleens and femurs were removed from two individuals from each group. Spleens were dissociated in sterile phosphate buffered saline solution (PBS pH 7.6) with foetal bovine serum (FBS, 10%) in sterile Petri dishes until obtention of single-cell suspensions. Splenic suspensions were centrifuged through a density gradient (Ficoll-Paque[®], GE LifeSciences) at 1500rpm for 15 minutes at room temperature. Polimorfonuclear fractions were collected to new tubes, centrifuged as above, diluted in 100 μ L of PBS+FBS and kept on ice. Inner septums of femurs were flushed in 2mL phosphate buffered saline solution (PBS pH 7.6) with foetal bovine serum (FBS, 10%) and EDTA (5mM). Bone marrow cell suspensions were washed in same solution by centrifugation (1500 rpm, 15min, RT), diluted as above and kept on ice. Splenic and bone marrow suspensions were, centrifuged and diluted in 200 μ L PBS+FBS and received 1 μ g/10⁶ cells of PE-conjugated anti-mouse CD34 clone MEC14.7 (SANTA CRUZ) and allowed to bind for 30min at 4°C away from light sources until event acquisition in a FACSCalibur unit (Becton Dickinson). Acquisitions were performed until 50000 events. Results were given in fold changes of CD34⁺ counts relative to non-irradiated controls. Data analyses were performed using Flowing 1.6.0 software (Turku Centre for Biotechnology, Finland). Relevant events were gated to proper FSC and SSC regions and FL2 fluorescence, using procedures described as Millan-Mullhouse protocol [30]. Samples from all described groups were evaluated by this method, except iNOS^{-/-}.

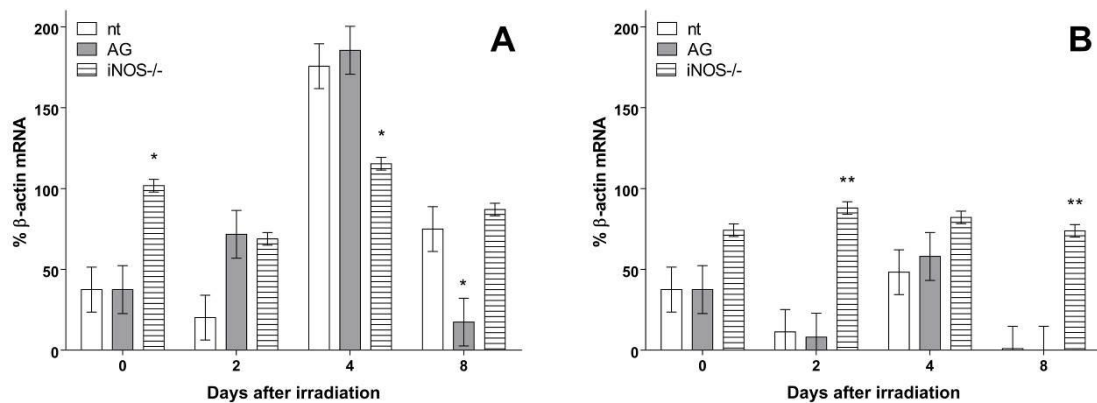
2.9. Statistical analysis

All collected data was analysed using Bonferroni tests (95% confidence intervals) to find differences between groups. All analyses were performed using GraphPad Prism 7.0 software package.

3. RESULTS AND DISCUSSION

Suppression of iNOS expression maintained *CXCL12* mRNA production constant during the experiment in hematopoietic organs: in Figure 2A it is shown *CXCL12* mRNA production relative to β -actin mRNA in spleen cells of 8Gy irradiated mice. AG treated mice showed higher levels of chemokine mRNA expression at day 2 after irradiation ($p < 0.01$) but lowered at day 8 ($p < 0.05$) in splenic polymorphonuclear fractions comparing to same day non-treated irradiated controls (nt).

Figure 1: *CXCL12* mRNA production in spleen (A) and bone marrow (B) cells from 8Gy irradiated groups. Data represents *CXCL12* % mRNA in percentage of β -actin mRNA production of samples in all days of experiment. (*) represents difference from same day control (nt; non-treated irradiated mice): $p < 0.01$. (**): $p < 0.05$. Error bars represent SEM from groups

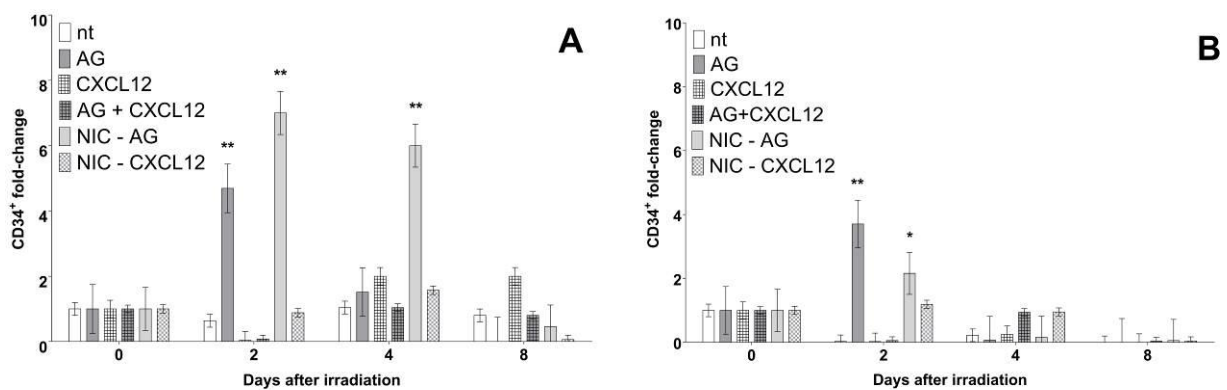


iNOS knockout mice showed higher expression levels at day 0 (before irradiation) and at day fourth after irradiation than controls. In bone marrow cells, *CXCL12* mRNA production was found even more constant during the experiment, as showed in Figure 2A. iNOS knockout group showed

higher level of chemokine expression at days 0 (before irradiation) ($p < 0.01$) and eightieth after irradiation ($p < 0.01$).

AG oral treatment increased $CD34^+$ fractions in spleens of irradiated mice: in Figure 2A it is shown fold changes in $CD34^+$ splenic proportions from irradiated groups. It was found that treated group presents a significantly higher fold change ($p < 0.001$) at day second than same day controls. Interestingly, non-irradiated aminoguanidine treated group showed even higher changes at days second ($p < 0.001$) and fourth ($p < 0.001$) after irradiation. Treated groups, irradiated (AG) or not (NIC-AG) showed significant differences from all other groups at days 2 and 4.

Figure 2: Fold changes relative to controls of $CD34^+$ counts in spleen (A) on bone marrow (B) cells of all groups. (*) represents difference from same day control (nt; non-treated irradiated mice): $p < 0.001$. (**) Error bars represent SEM from groups.

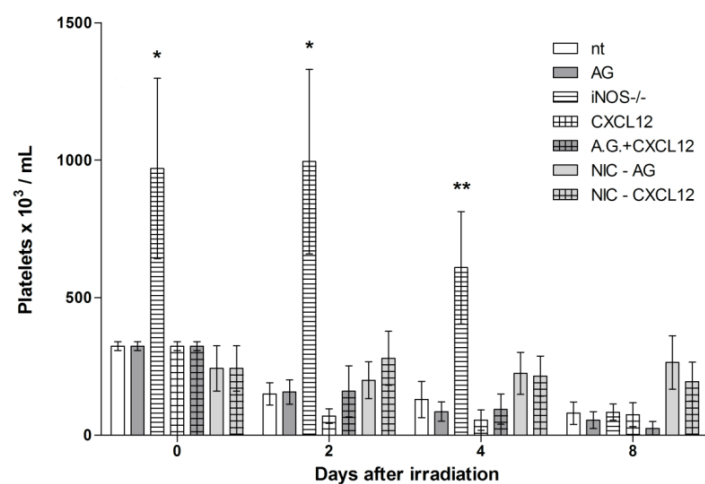


$CD34^+$ fold changes in bone marrow counts of irradiated mice were responsible for iNOS inhibition in a similar way comparing to spleen tissue: in Figure 2B it is shown that, similarly to spleen, higher fold changes in $CD34^+$ fraction at day 2 after irradiation occurred in bone marrow tissue ($p < 0.001$). Non-irradiated treated groups also showed significantly higher levels than controls at same day ($p < 0.01$). As observed in spleen cells, $CD34^+$ fold changes of treated (AG) and non-irradiated but treated groups (NIC-AG) were found significantly higher levels than controls at the same day ($p < 0.01$).

iNOS knockout mice showed higher platelet counts comparing to all other groups: in Figure 3 it is shown platelet counts from peripheral blood of experimental groups. iNOS knockout mice

showed significantly higher levels of platelet counts at days 0 ($p < 0.001$), 2 ($p < 0.001$) and 4 ($p < 0.05$) of experiment comparing to controls. $iNOS^{-/-}$ group showed higher platelet counts comparing to all other experimental groups.

Figure 3: Platelet counts from all experimental groups during all days of experiment. (*) represents difference from same day control (nt; non-treated irradiated mice): $p < 0.001$. (**): $p < 0.05$. Error bars represent SEM from groups.



Radiation induced hematopoietic tissue ablation was decreased by $iNOS$ suppression of expression: in Figures 4 and 5 are shown images of preparations of spleen tissue from non-treated (nt), aminoguanidine treated (AG) and $iNOS^{-/-}$ mice. No relevant visual differences could be perceived between histological preparations from groups through 10 or 40X magnifications. Brown-colored artifacts were found to be related to hemosiderin presence.

In figures 6 and 7 are shown femoral bone marrow preparations from same groups. Preparations from $iNOS$ -suppressed groups showed less tissue destruction and more densely populated niches, more evident in preparations from knockout mice at day 8 after irradiation. In all analysed tissues, $iNOS$ genomic knockout was more effective to prevent tissue destruction than its chemically-treated counterpart.

Figure 4: Histological images of spleen preparations (HE stain) from non-treated, treated (aminoguanidine) and *iNOS*^{-/-} 8Gy irradiated mice. Numbers on left refers to days after irradiation. Visualization: 10X. Bar: 200µm. White arrows: white pulp. Red arrows: red pulp. Green arrows: hemosiderin.

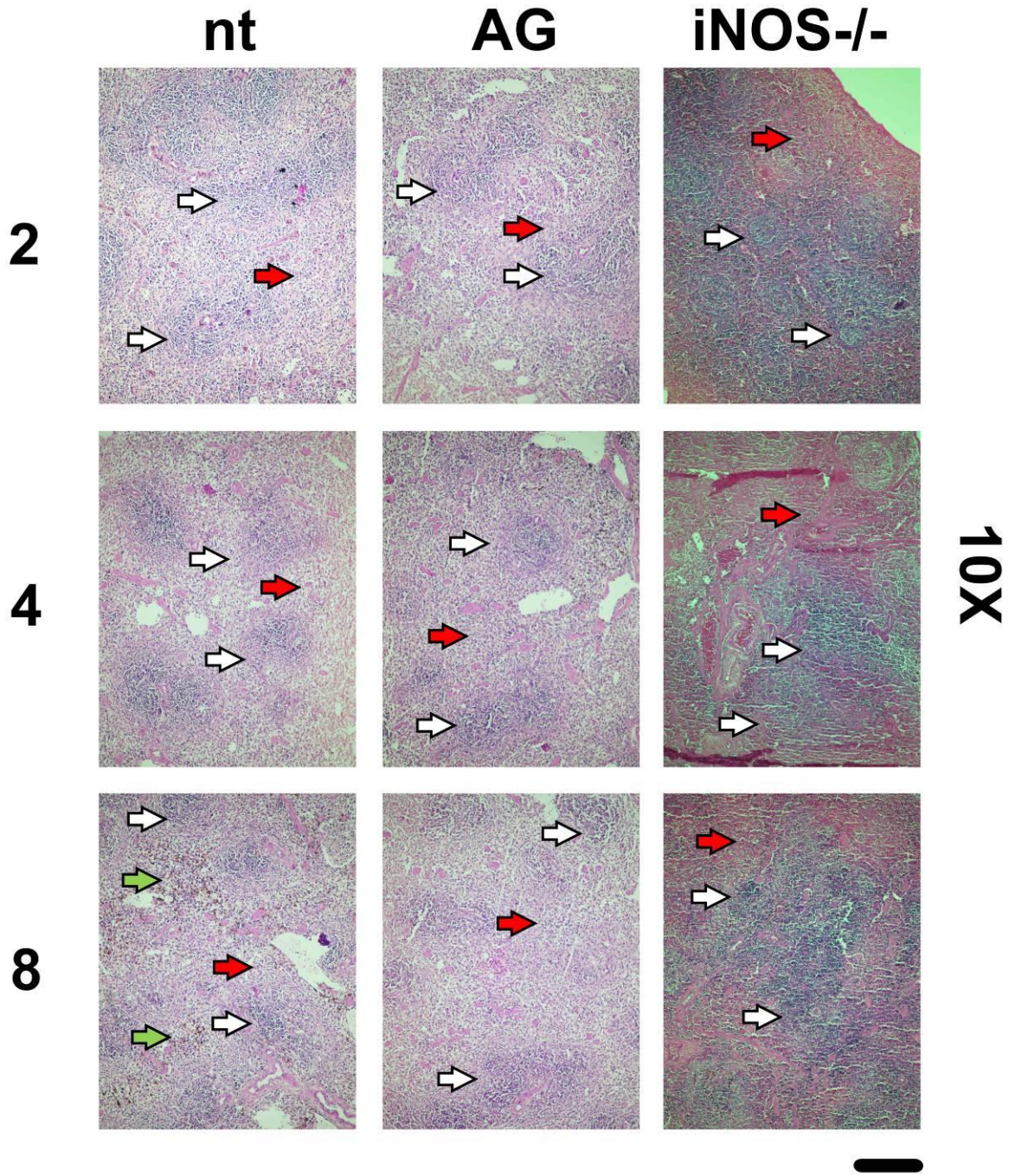


Figure 5: Histological images of spleen preparations (HE stain) from non-treated, treated (aminoguanidine) and *iNOS*^{-/-} 8Gy irradiated mice. Numbers on left refers to days after irradiation. Visualization: 40X. Bar: 50µm. White arrows: white pulp. Red arrows: red pulp. Green arrows: hemosiderin.

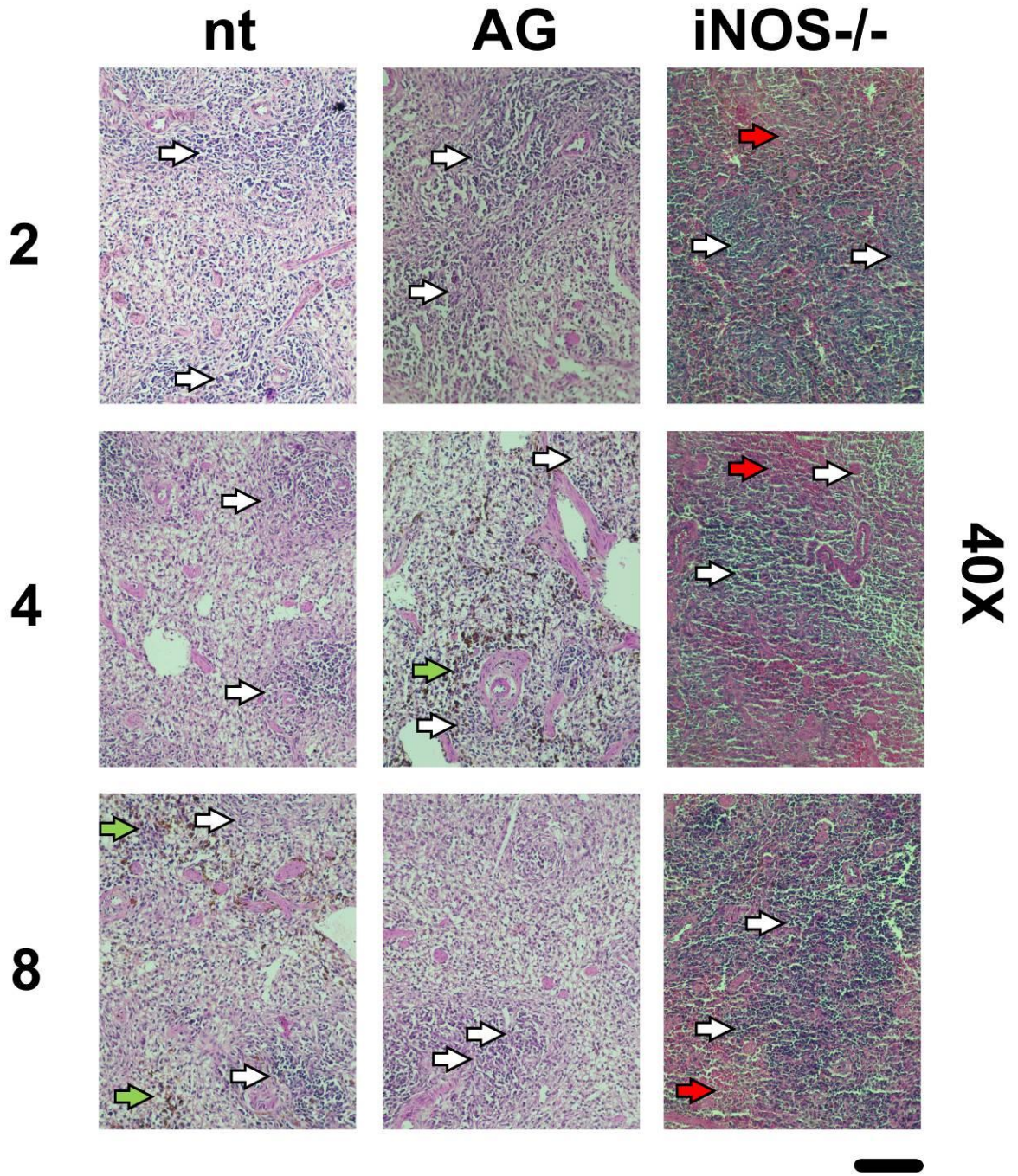


Figure 6: Histological images of bone marrow preparations (HE stain) from non-treated, treated (aminoguanidine) and *iNOS*^{-/-} 8Gy irradiated mice. Numbers on left refers to days after irradiation. Visualization: 10X. Bar: 200µm. White arrows: nucleated cells. Red arrows: erythrocytes. Green arrows: bone matrix.

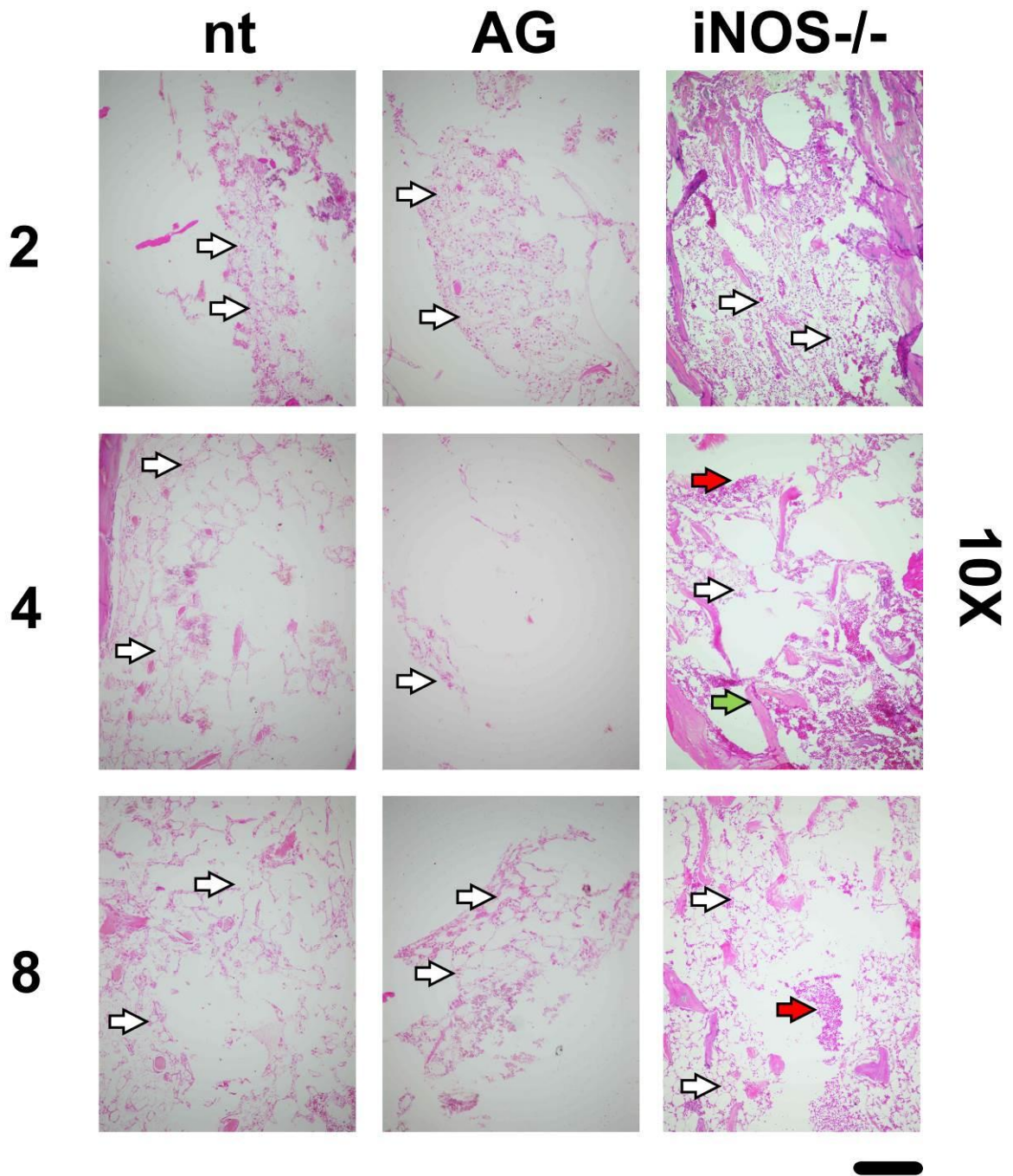
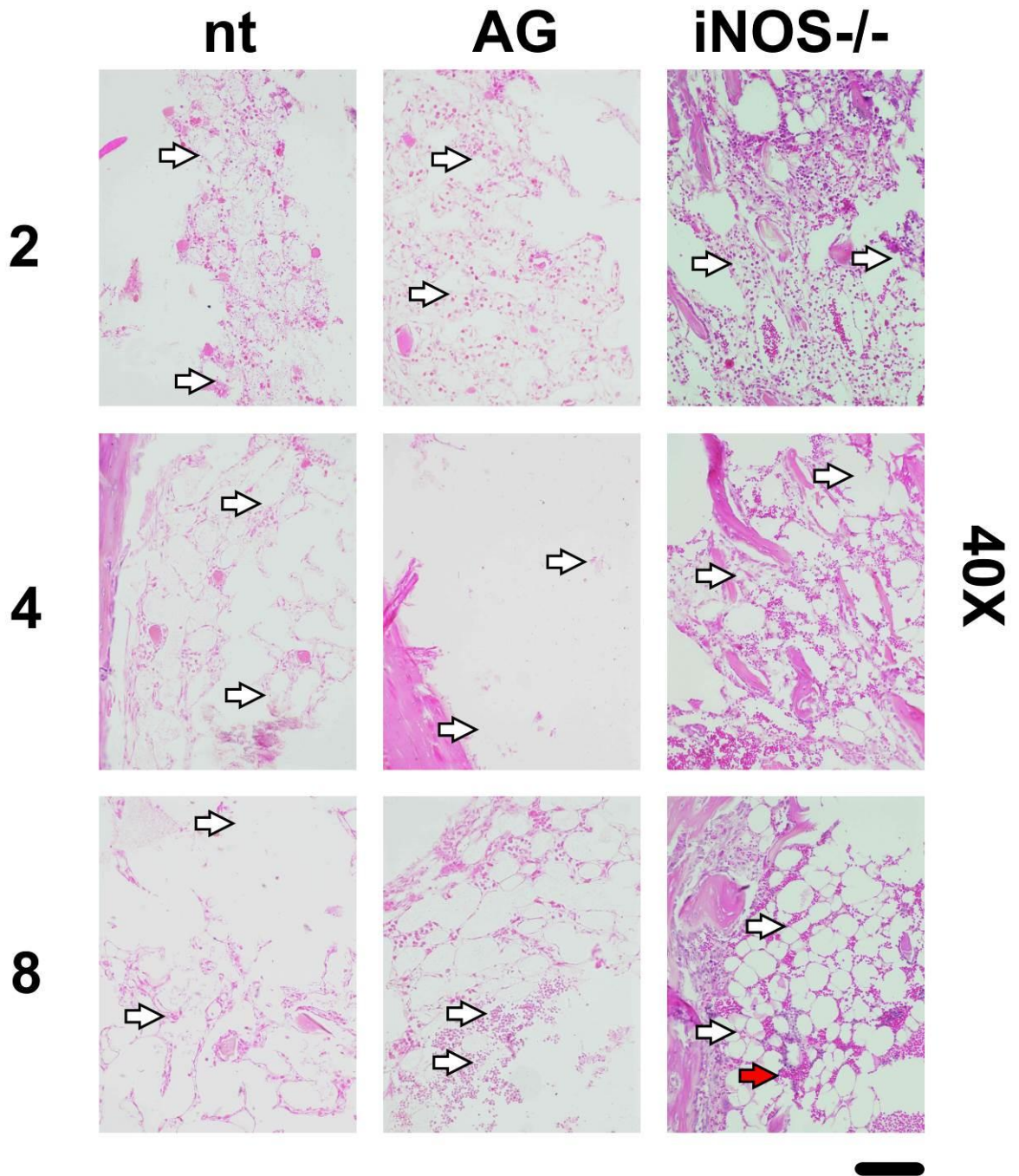


Figure 7: Histological images of bone marrow preparations (HE stain) from non-treated, treated (aminoguanidine) and *iNOS*^{-/-} 8Gy irradiated mice. Numbers on left refers to days after irradiation. Visualization: 40X. Bar: 50µm. White arrows: nucleated cells. Red arrows: erythrocytes.



In this study AG was orally administered in mice to prevent cell death in irradiated tissues, and IR-induced hematopoietic tissue destruction, CD34⁺ cell recruitment to hematopoietic sites and its possible effects on platelet counts were evaluated. This work also aimed to find a relationship between iNOS inhibition and *CXCL12* mRNA expression in these organs as found in models cited above.

After IR exposures, disseminated tissue destruction and increasing of necrosis and apoptosis frequencies are expected in hematopoietic tissues [31]. In rodent models, bone marrow and spleen are the major hematopoietic organs. Bone marrow tissue and cell populations are globally more radiosensitive than in splenic populations due to its greater mitotic index. In the latter case, higher counts of phagocytic cells as dendritic CD11c⁺ and CD11b⁺ [32, 33], and larger concentration of erythrocytes due to a vast blood flow inside this organ can give this tissue more global radio resistance than bone marrow. These cells display relevant levels of differentiation and, consequently, less proliferation capability. Bone marrow cell populations are composed in majority by stem-cells with very high proliferation capacities, and thus much more susceptible to IR-exposure damaging events despite their localization inside compact bones as femurs and vertebrae.

Aminoguanidine is a selective iNOS inhibitor and its inhibition effects are much less remarkable on nNOS and eNOS isoforms [34]. Upon decreased NO production, less peroxynitrite (ONOO⁻) can be produced after reaction with intracellular peroxides, thus reducing concentration of oxidative radicals inside cellular compartments, and diminishing some deleterious effects after IR exposures. In distinct experimental models, iNOS expression could be related to increase in *CXCL12* chemokine expression after NO release in splenic tissue in mice experimentally infected with a non-lethal strain of rodent malaria [29]. In the same models, *CXCL12* is strongly related to immune system cell proliferation after infection [35]. In IR exposure studies, *CXCL12* expression is related to bone marrow stem-cell homing [36]. In the same way, *CXCL12* supplementation in irradiated experimental models can actively help recruitment of stem cells from peripheral blood and homing of these cells in hematopoietic microenvironments. In these cases, administration of *CXCL12* in experimental models allows the use of fewer transplanted stem cells, or to decrease the period of "nadir" of the receiver [37].

Synthetic murine *CXCL12* intraperitoneally administered was found to be ineffective in altering platelet counts in the present experiments. Groups that received chemokine injections (*CXCL12*)

and also AG in drinking water (AG+*CXCL12*) did not develop statistically significant differences in platelet counts. The reduction of nitric oxide presence, caused by the functional blockade of its synthesis, or by gene knockout strategies, is known to reduce the adhesion and aggregation capacity of platelets [38, 39], possibly due to a signalling by cGMP-dependent protein kinase I (cGKI) [40]. If the NO absence impairs these processes, it could be possible to assume that, due to lesser extents of endothelial adhesion, circulating levels of platelets could be increased in iNOS^{-/-} mice. Also, the iNOS knockout increases vascular permeability, what could increase the number of platelets in circulating blood [41].

In spleen cells, *CXCL12* could not increase or even sustain CD34⁺ populations in irradiated tissues. Conversely, in bone marrow suspensions from irradiated *CXCL12*-treated groups it was found that chemokine administration reduced the proportion of these cells. Additionally, *CXCL12* acts also as an antiapoptotic factor in hematopoietic stem cells, as found in other work [42], what could lead to expectation that *CXCL12* administration might increase recruitment from peripheral blood and homing at bone marrow of CD34⁺ populations. In an inverse model, *CXCL12* administration can induce MMP-2 and MMP-9 (matrix metalloproteinases 2 and 9) expression by CD34⁺ cells, what is enough for detachment of these cells from periosteum and subsequent escape from bone marrow environment to peripheral blood [43]. Probably the administered *CXCL12* dose (1µg/kg at second, fourth and eightieth days after irradiation) was sufficiently high to trigger this kind of response.

Suppression of iNOS expression seemed to be an important factor to keep *CXCL12* mRNA expression during the experiments. AG-treated mice showed significant higher levels of *CXCL12* mRNA expression in spleen cells until up to four days after irradiation with 8 Gy, but the most impacting data came from the iNOS knockout group, that kept chemokine mRNA levels constant in both organs during the experiment, but more consistent in bone marrow. The sustained *CXCL12* mRNA expression could explain higher platelet counts in knockout group (8Gy irradiated), once *CXCL12* expression is related to upregulation of PDGF-β (Platelet-derived growth factor – beta), an important cytokine that attracts megakaryocytes to bone marrow stem cell niches [44].

CXCL12 mRNA presence was considered constant through duration of experiment in spleens and bone marrows of iNOS knockout mice and showed to be significantly different than controls at days 0 and 4 (spleens) and 2 and 8 (bone marrows). AG administration reduced chemokine mRNA

levels only in spleens 8 days after irradiation. Despite numerical differences, no statistically significant differences between groups could be observed in spleens at day 2 after irradiation. In bone marrow, this chemokine is related to be expressed by vascular, perivascular and osteogenic cells [45], which are cell subtypes not classically related to be hematopoietic blood components-forming stem cells. Endothelial CD31⁺ cells are associated to be responsible for hematopoietic niche regeneration after radiation injury [45], leading to an interpretation in that these cell subtypes can resist to radiation damage to some extent. The presence of nitrogen-associated radicals in hematopoietic sites can reduce *CXCL12* activity through nitration [46], and in inflammatory models, nitric oxide presence was shown to reduce *CXCL12* gene expression [47]. In this manner, it could be possible to assume the NO absence as a putative factor of *CXCL12* mRNA expression maintenance in constant levels, as showed by the work.

After exposures to 4Gy, the present working concentration of orally administered AG led to no visible effect on preventing tissue destruction in spleens and bone marrow from mice groups (data not shown). Treated, non-treated and iNOS knockout groups showed similar ablation levels in both organs based on visualization. In groups irradiated with 8Gy, it was possible to see a more evident increasing in bone marrow cellularity in treated and knockout groups, especially at the second day after irradiation, and less evident effects at day 4. AG seemed to have little protective effect while analysing the histological samples. More objective approaches could only be carried out using specific apoptosis and necrosis detection to show disseminated cell death after IR exposure.

The microscopic observation showed that, at the day 4 after irradiation, bone marrow from AG-treated group exhibits only sparse tissue strings, indicating limited tissue regeneration and/or cell proliferation. At day 8, bone marrow from nt and AG groups seemed to be in quantitatively comparable states of regeneration. iNOS-deficient mice showed significant number of regeneration sites, characterized by cell strings, probably due to increased vascular permeability associated to the lack (or the reduction) of NO at hematopoietic sites [41]. The presence of erythrocytes only in bone marrow preparations of knockout mice also aid this interpretation. In addition to the sustained *CXCL12* mRNA presence, indicating its constant expression, this permeability may have helped in bone marrow regeneration. Aminoguanidine administration could not induce this effect, apparently. Alternatively, histological analysis of bone marrow of AG-treated groups showed even lower cellularity than in controls, principally four days after irradiation. Another nitric oxide production inhibitor (L-

NMMA) is found to be hematotoxic, and a relevant contributor to cellularity loss in other animal models of bone marrow depletion [48]. In this case, the authors could interpret that AG induced some toxicity, at least to a limited extent, until the day 4 of experiment. In this same day, *CXCL12* mRNA presence in spleens reached its maximum value during the experiment. Considering the role of this chemokine in homing of stem cells in hematopoietic sites, and the fact that the splenic sites were showed as well-populated and in a relatively positive state of regeneration, one possible thesis could be that the hematopoietic cells from bone marrow were attracted to splenic sites. Non-irradiated and irradiated AG-treated mice showed increased CD34⁺ proportions in spleens, what could illustrate this phenomenon, despite not enough to keep this migratory influx and further homing after radiation-mediated depletion. Intraperitoneal injection of synthetic *CXCL12* not helped in CD34⁺ cell homing at hematopoietic sites, even in a relatively high dose. As discussed before, the high dose probably acted as a detachment factor from hematopoietic sites [43], and the escape from bone marrow could be enhanced through the already cited increased in vascular permeation.

Regarding CD34⁺ counts in hematopoietic organs, AG administration induced significant increase in recruitment of this cell population in spleen and bone marrow of treated mice irradiated at 8Gy, more clearly at day 2 after irradiation. It was possible to find increased numbers of CD34⁺ counts in the same organs at day 2 after irradiation compared to controls. Other studies worked on clarify whether iNOS/NO suppression can increase survival of high mitotic rate cell types [49]. Aminoguanidine-based iNOS inhibiting and protective effects in CD34⁺ pooled fractions could only be significantly observed in 8Gy irradiated groups, what can be a concentration effect. Studies are discrepant when arguing whether IC_{50%} values for AG administration [50] are effective. Relatively to low specificity of aminoguanidine and other iNOS activity are indicative that higher concentrations must be tested. Accordingly, the present experimental design was not able to reveal differences between treated and non-treated groups in *CXCL12* mRNA expression sampled in spleen and bone marrow tissues of irradiated mice. However, AG orally administered could increase CD34⁺ counts in spleens from 8Gy irradiated at days 2 and 4 after irradiation, and in bone marrow at day 2, suggesting that the employed concentration could induce significant changes comparing to same day control.

The work did not search for CD34⁺ cell populations in iNOS^{-/-} mice, but other studies of tumoral models using same knockout mice showed increase of frequencies of endothelial CD34⁺ in

osteosarcoma [51] in squamous cell carcinoma [52] and in hepatocellular carcinoma [53]. Also, CD34⁺ monocytes from iNOS^{-/-} mice retain enhanced viability potential [54]. Thus, the scientific evidence of CD34⁺ survival in iNOS depleted mice can be taken as positive and was assumed as true by this work.

4. CONCLUSION

iNOS genetically knocked-out mice are less susceptible to IR-induced tissue damage, and kept *CXCL12* mRNA expression levels constant up to eight days after irradiation. To reproduce this effect, oral administration of aminoguanidine was provided to irradiated mice, resulting in non-evident histological modifications (by simple HE-staining). On the other hand, a significant increase in progenitor CD34⁺ counts were seen to occur in treated groups, despite no hematological modification could be observed among the experimental groups. The results suggest that iNOS suppression was found to be related to cellular repopulation of hematopoietic sites in mice irradiated by lethal doses.

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