Published in final edited form as:

Plast Reconstr Surg. 2019 June ; 143(6): 1666–1676. doi:10.1097/PRS.0000000000005647.

“The Role of Deferoxamine in Irradiated Breast Reconstruction: A Study of Oncologic Safety”

Jeremy V. Lynn, BS¹, Kevin M. Urlaub, BS¹, Kavitha Ranganathan, MD¹, Alexis Donneys, MD, MS¹, Noah S. Nelson, BS¹, Chitra Subramanian, PhD, MBA², Mark S. Cohen, MD, FACS^{2,3}, and Steven R. Buchman, MD¹

¹Department of Surgery, Section of Plastic and Reconstructive Surgery, University of Michigan, Ann Arbor, Michigan.

²Department of Surgery, University of Michigan, Ann Arbor, Michigan.

³Department of Pharmacology, University of Michigan, Ann Arbor, Michigan.

Abstract

Background: Radiotherapy plays an essential role in the oncologic management of breast cancer. However, patients who undergo radiotherapy experience significantly more wound complications during the reconstructive process. Deferoxamine is an FDA-approved iron chelator with immense potential to up-regulate angiogenesis and improve reconstructive outcomes. The purpose of this study is to determine the impact of deferoxamine on breast cancer cell proliferation *in-vitro*, to delineate oncologic safety concerns regarding the utilization of deferoxamine as a regenerative therapeutic.

Methods: The dose-dependent effect of radiation and deferoxamine on two triple-negative breast cancer cell lines (MDA-MB-231 and MDA-MB-468) was determined via MTS (percent cell viability) and tumorsphere (sphere number) analysis. Radiation (0, 5, and 10Gy) and deferoxamine (0, 25, 50, 75, and 100μM) were delivered both individually and in combination, and all experiments were completed in triplicate. Intracellular iron, NF-κB localization, and apoptosis/necrosis assays were performed to delineate mechanism. ANOVA statistical analysis was performed using SPSS ($p<0.05$).

Results: For both cell lines, percent viability and sphere number significantly decreased following exposure to 10Gy of radiation. Surprisingly, the administration of 25μM deferoxamine also significantly decreased each metric. The administration of deferoxamine (100μM) in combination with radiation (10Gy) resulted in significantly reduced percent viability and sphere

Corresponding Author: Steven R. Buchman, MD, University of Michigan, 1540 E. Hospital Dr., Floor 4, Suite 4-730, Ann Arbor, Michigan 48104 USA, sbuchman@med.umich.edu.

AUTHOR CONTRIBUTIONS

All authors contributed to the design and execution of this study, as well as composition and refinement of this manuscript. Specifically, Jeremy Lynn, BS directly oversaw all components of this work in the laboratory and wrote the first draft of the paper. Kevin Urlaub, Kavitha Ranganathan, MD, Alexis Donneys, MD, and Noah S. Nelson, MPH contributed to the *in-vitro* work and edited the first draft of the paper. Finally, Chitra Subramanian, PhD, MBA, Mark S. Cohen, MD, FACS, and Steven R. Buchman, MD provided senior guidance and support both in the laboratory space and in the drafting and finalization of this letter.

Financial Disclosure Statement: The authors have nothing to disclose.

number compared to the administration of radiation alone. Deferoxamine treatment decreased intracellular iron, suppressed NF- κ B activation, and induced apoptosis.

Conclusions: Radiation and deferoxamine significantly decrease breast cancer cell proliferation when delivered independently and in combination, suggesting deferoxamine may be safely utilized to facilitate improved reconstructive outcomes among triple-negative breast cancer survivors.

Keywords

Triple-Negative Breast Neoplasms; MDA-MB-231; MDA-MB-468; Iron chelation; Angiogenesis

INTRODUCTION

An estimated 316,000 new cases of invasive or *in situ* breast cancer will be diagnosed among females in the United States in 2018.¹ Depending on the stage of cancer at diagnosis, oncologic treatment may involve breast-conserving surgery or mastectomy.² While subsequent administration of radiotherapy is known to substantially reduce breast cancer recurrence and absolute mortality by destroying residual cancer cells, the acute and long-term side effects of radiotherapy on surrounding soft tissue warrant consideration.³⁻⁴ Notably, radiation disrupts healthy cell function, destroys vascular networks, and severely inhibits angiogenesis.⁵ The resultant skin and soft tissue atrophy challenges the reconstructive process and ultimately diminishes overall aesthetic outcomes and patient satisfaction.⁶ Considering both the current irreplaceable role of radiation in oncologic management and the marked importance of breast reconstruction to patients' overall recovery, translational plastic and reconstructive surgery research must continually pursue therapeutic treatments for radiation-induced skin and soft tissue injury.

Deferoxamine, an FDA-approved iron chelator, was first developed for the systemic treatment of hemochromatosis.⁷ This pharmacologic agent has since been studied in the context soft tissue healing and regeneration. In 2015, Mericli et al. delineated the ability of deferoxamine to alleviate radiation-induced hypovascularity and improve tissue elasticity in a murine transverse rectus abdominis myocutaneous flap model.⁸⁻⁹ Duscher et al. then demonstrated the ability of deferoxamine to aid in diabetic wound healing in 2015, and extended this finding to aged wound healing in 2017.¹⁰⁻¹¹ Most recently, Flacco et al. demonstrated increased fat graft retention for soft-tissue reconstruction following radiotherapy when tissue was pre-treated with deferoxamine.¹²

Alongside the aforementioned breakthroughs, our laboratory has developed a murine model of expander-based breast reconstruction and investigated the capacity of pharmaceutical therapies such as deferoxamine to aid in post-radiation tissue expansion and breast reconstruction.¹³⁻¹⁵ Our initial studies suggest deferoxamine is capable of reducing skin ulceration and restoring type I collagen fibril disorganization following radiotherapy. These promising results are most likely attributable to the angiogenic potential of deferoxamine.¹⁶ Through local iron chelation, deferoxamine stimulates the HIF-1 α pathway. The elevated VEGF production in turn promotes the formation of healthy vascular networks.¹⁷ Notwithstanding, there remains legitimate concern regarding the administration of an

angiogenic stimulant such as deferoxamine in close proximity to potentially cancerous breast tissue.

Several *in-vitro* and *in-vivo* studies have been performed to determine the impact of iron chelation on cancer proliferation and metastasis.¹⁸ Some investigations suggest the administration of iron sequestering agents poses significant risk to patients with cancer due to increased HIF-1 α and VEGF expression.¹⁹ Many others posit such pharmaceutical agents may actually be utilized as chemotherapeutic strategies given the potential for disruption of various metabolic pathways required for tumor growth.²⁰⁻²⁵ Based on the relative lack of consensus in the scientific literature, and the absence of studies examining potential synergistic or antagonistic effects of deferoxamine and radiation when administered jointly, a more complete investigation of the impact of deferoxamine on breast cancer proliferation is urgently warranted. The purpose of this study is to determine the impact of deferoxamine delivered independently and in combination with radiation on triple-negative breast cancer cell proliferation *in-vitro* in order to delineate oncologic safety concerns regarding the utilization of deferoxamine as a regenerative therapeutic in irradiated breast reconstruction.

MATERIALS AND METHODS

Cell Culture and Chemicals

Two validated triple-negative breast cancer cell lines (MDA-MB-231 and MDB-MB-468), one estrogen and progesterone positive cell line (MCF-7), and one normal cell line (female fibroblast, FF) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 100X antibiotic-antimycotic (Sigma-Aldrich) and 10% fetal bovine serum (Sigma-Aldrich). Cells were incubated in a 37°C humidified atmosphere of 5% CO₂ in air. Triple-negative breast cancer cells, which lack estrogen receptors (ER-) and progesterone receptors (PR-) as well as human epidermal growth factor 2 (HER2-), were primarily utilized in this study because these cell lines do not respond to hormone or HER2 blocking therapies. Therefore, patients with triple-negative breast cancer commonly receive chemoradiation and are potential candidates to receive deferoxamine as a regenerative therapeutic during the reconstructive process.²⁶ MCF-7 cells were studied secondarily to broaden the applicability of this study, as the behavior of breast cancer cells to radiation and deferoxamine is expected to vary depending on receptor status.²⁷ Finally, FF cells were studied to determine the susceptibility of normal human cells to radiation and deferoxamine treatment relative to cancer cells. Deferoxamine mesylate was obtained from Desferal; Hospira, Lake Forest, USA.

Therapeutic Dosing

Cells were plated and allowed 24 hours to proliferate. A single dose of 0, 5, or 10 Gy of radiation was then administered to select experimental groups using a Philips RT250 orthovoltage unit (Kimtron, Inc., Oxford, CT). The dose range of 0 to 10 Gy was selected based on previously established protocols proven to significantly inhibit the growth and development of triple-negative breast cancer cells.²⁸⁻³⁰ Furthermore, deferoxamine was dissolved in sterile deionized water and added to select experimental culture wells to achieve a final concentration of 0, 25, 50, 75, or 100 μ M immediately following radiation

administration. The dose range of 0 to 100 μ M was chosen based on several published studies that demonstrate the ability of deferoxamine to inhibit triple-negative breast cancer cell proliferation.^{23, 31-32} Of note, previous *in-vitro* investigations of deferoxamine in our laboratory verify the abovementioned dose range is not simply toxic to all cell lines, but may actually promote the growth of certain cell lines such as human umbilical vein endothelial cells of FF cells.³³

Analysis of Percent Cell Viability via MTS Assay

Percent cell viability was evaluated using the CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay (Promega, Fitchburg, WI). Approximately 2,500 cells (MDA-MB-231, MDA-MB-468, MCF-7, or FF) were seeded in 96-well micro titer plates in 100 μ L of DMEM and allowed 24 hours to adhere. Cells were then dosed with radiation and/or deferoxamine in replicates of three as described above. Following treatment, the number of viable cells was evaluated by utilizing a BioTek Synergy Neo Plate Reader (BioTek, Winooski, VT, USA) to measure A490 of the dissolved formazan reagent after the addition of 20 μ L of the MTS reagent for 2 hours as per the manufacturer's protocol. Percent viability was ultimately calculated as the ratio of viable cells within each treatment group compared to the control group.

Analysis of 3-Dimensional Cell Growth via Tumorsphere Formation Assay

MDA-MB-231 and MDA-MB-468 cells were plated at 250 cells per well in 96-well ultralow attachment plates (Corning, Corning, NY) in 100 μ L of MammoCult[™] human media (STEMCELL Technologies, Vancouver, Canada). Twenty-four hours after being plated, cells received radiation and/or deferoxamine depending on the experimental group. Tumorsphere formation was evaluated using an EVOS FLC microscope (Thermo Fisher Scientific, Carlsbad, CA) 7 days after therapeutic dosing and reported as sphere number.

Analysis of Intracellular Iron via QuantiChrom[™] Assay

A Quantichrom[™] Iron Assay Kit (DIFE-250, BioAssay Systems, Thermo Fisher Scientific, Carlsbad, CA) was utilized to determine the impact of deferoxamine on intracellular iron levels. Briefly, MDA-MB-231 and MDA-MB-468 cells were plated at 500,000 cells per well in 1.5 mL of DMEM in 6-well plates and allowed 24 hours to adhere. Cells were then dosed with deferoxamine for 24 hours prior to analysis as per the manufacturer protocol. Protein content was measured via BCA assay for each sample. Intracellular iron concentration was measured using a QuantiChrom[™] Assay. The values were standardized to μ g of iron per μ g of protein. These values were then calibrated to represent percent change in iron concentration compared to control. Each experiment was carried out in triplicate and the values are presented as mean $\pm$ standard deviation.

Analysis of NF- κ B Activation via Dual-Luciferase Reporter Assay

Nuclear factor- κ B (NF- κ B) activation was measured via immunohistochemical analysis of NF- κ B localization. Briefly, MDA-MB-231 and MDA-MB-468 cells were plated at 25,000 cells per well in 450 μ L DMEM in 8-well glass Lab-Tek[™] II Chamber Slide[™] System slides (Thermo Fisher Scientific, Carlsbad, CA) and allowed 24 hours to adhere. Then,

thirty-six hours after therapeutic dosing with radiation and/or deferoxamine, cells were fixed with 4% paraformaldehyde, permeabilized with 1% Triton X100, and blocked with 5% NGS. The cells were incubated with primary P65 antibody for 24 hours, washed, and then treated with fluorescent-labeled secondary antibodies for 1 hour at room temperature. Cells were washed and mounted on a cover slide.

Analysis of Apoptosis Versus Necrosis via Annexin V Assay

A RealTime Glo™ Annexin V Apoptosis and Necrosis Assay (Promega, Fitchburg, WI) was utilized to investigate the mechanism of cell death caused by radiation and deferoxamine. Briefly, MDA-MB-231 and MDA-MB-468 cells were plated at 10,000 cells per well in 40 uL of DMEM in white tissue-culture treated 96-well plates (Corning, Corning, NY) and allowed 24 hours to adhere. Radiation and deferoxamine were administered as described above, and 50 uL of detection agent was added and apoptosis/necrosis were measured as per the manufacturer's instruction. A BioTek Synergy Neo Plate Reader was utilized to quantify luminescence and fluorescence at the following time points: 0, 2, 4, 6, 8, 24, and 48 hours. These time points were determined based on live cell imaging studies that demonstrated the toxicity of radiation and deferoxamine just 24 hours after administration.

Cell Imaging

Bright field images were taken using an EVOS FLc microscope. To qualitatively analyze cell proliferation, fluorescent images were captured using MDA-MB-468 cells expressing a red fluorescent protein tag (tdTomato), which was generated by lentiviral transduction followed by selection with 0.5 ug/mL puromycin (Sigma-Aldrich) in DMEM. Fluorescent images were also taken using an EVOS FLc microscope. Live cell imaging was performed by the University of Michigan Microscopic Video Surveillance Core.

Statistical Analysis

All experiments were performed in triplicate. Significance ($p < 0.05$) between groups was identified by One-Way ANOVA analysis performed using SPSS v24 (SPSS, INC., Chicago, Ill). Unless otherwise noted, data values are presented as mean $\pm$ standard error.

RESULTS

Radiation Inhibits Triple-Negative Breast Cancer Cell Proliferation and 3-Dimensional Tumorsphere Formation

Two distinct *in-vitro* assays were performed to confirm the well-established toxicity of radiation to two triple-negative breast cancer cells (Figure 1, above). An MTS assay, which was performed to measure the cellular metabolic activity, demonstrated the toxicity of 10 Gy of radiation to both cell lines as evidenced by a significant reduction in percent cell viability (MDA-MB-231: 77% versus 100%, $p=0.000$; MDA-MB-468: 76% versus 100%, $p=0.048$). Receptor-positive MCF-7 cells examined to expand the applicability of this study demonstrated a significant decrease in cell viability following exposure to 5 Gy (84% versus 100%, $p=0.000$; See Figure, Supplemental Digital Content 1, which shows the percent cell viability of MCF-7 cells following exposure to 0, 5, or 10 Gy of radiation (left); 0, 25, 50, 75, or 100 μ M of deferoxamine (middle); and 10 Gy of radiation and 0, 25, 50, 75, or 100

μM of deferoxamine (right). Respectively, statistical significance relative to 0 Gy, 0 μM, and 10 Gy plus 0 μM was determined via one-way ANOVA (* $p < 0.05$, ** $p < 0.01$). Error bars represent standard error). Noncancerous FF cells were examined as a control and did not demonstrate a significant response to 10 Gy (90% versus 100%, $p = 0.783$). A 3-dimensional tumorsphere assay was then performed to simulate *in-vivo* breast cancer tumor formation.³⁴ The administration of 5 Gy of radiation resulted in a significantly reduced sphere number for both cell lines compared to control (MDA-MB-231: 14 versus 29, $p = 0.000$; MDA-MB-468: 9 versus 27, $p = 0.000$). These results conservatively demonstrate that 10 Gy of radiation inhibits triple-negative breast cancer cell proliferation.

Deferoxamine Inhibits Triple-Negative Breast Cancer Cell Proliferation and 3-Dimensional Tumorsphere Formation

To evaluate the potential effects of deferoxamine on triple-negative breast cancer cells when administered independently, two *in-vitro* studies were performed (Figure 1, below). Interestingly, the administration of 25 μM of deferoxamine significantly decreased the percentage of viable cells in culture compared to control, as determined by an MTS assay (MDA-MD-231: 84% versus 100%, $p = 0.003$; MDA-MB-468: 56% versus 100%, $p = 0.000$). Receptor positive MCF-7 cells demonstrated a comparable response to 25 μM of deferoxamine (65% versus 100%, $p = 0.000$; See Figure, Supplemental Digital Content 1). FF control cells demonstrated a non-significant trend towards increased proliferation following deferoxamine administration (100 μM: 110% versus 100%, $p = 0.729$). A 3-dimensional tumorsphere assay was then performed, and the administration of 25 μM of deferoxamine resulted in the formation of significantly less spheres compared to control (MDA-MD-231: 15 versus 29, $p = 0.000$; MDA-MB-468: 11 versus 27, $p = 0.000$). Taken together, these results suggest 25 μM of deferoxamine inhibits triple-negative breast cancer cell proliferation *in-vitro*.

Deferoxamine and Radiation Cooperatively Disrupt Triple-Negative Breast Cancer Proliferation and 3-Dimensional Tumorsphere Formation

To identify oncologic safety concerns surrounding the administration of deferoxamine to patients undergoing irradiated breast reconstruction, we sought to delineate the impact of deferoxamine on the toxicity profile of radiation therapy, a well established therapeutic for triple-negative breast cancer. Cells in culture were administered 10 Gy of radiation and 0, 25, 50, 75, or 100 μM of deferoxamine (Figure 2, above). Based on MTS analysis, the administration of 100 μM of deferoxamine in addition to 10 Gy of radiation decreased the percent viability of cells to a significantly greater degree than the administration of 10 Gy of radiation alone (MDA-MB-231: 69% versus 77%, $p = 0.010$; MDA-MB-468: 54% versus 76%, $p = 0.037$). MCF-7 cells responded similarly to 50 μM and 10 Gy compared to 10 Gy alone (56% versus 73%, $p = 0.003$; Supplemental Digital Content 1). Notably, deferoxamine protected non-cancerous FF cells from radiation, as evidenced by a significantly increased percent cell viability following 25 μM (113% versus 90%, $p = 0.023$). Moreover, the administration of 75 μM of deferoxamine in conjunction with 10 Gy of radiation inhibited the formation of tumorspheres to a significantly greater degree than the administration of 10 Gy alone (MDA-MB-231: 4 versus 8, $p = 0.002$; MDA-MB-468: 2 versus 6, $p = 0.010$). These

results demonstrate that deferoxamine and radiation work cooperatively to inhibit the viability of triple-negative breast cancer cells (Figure 2, below).

Deferoxamine Decreases Intracellular Iron in Triple-Negative Breast Cancer Cells

Intracellular iron concentration was investigated to begin delineating the mechanism by which deferoxamine inhibits triple-negative breast cancer proliferation (Figure 3, above). MDA-MB-231 and MDA-MB-468 cells demonstrated significantly reduced intracellular iron concentrations following exposure to 100 μ M and 75 μ M of deferoxamine for 24 hours compared to control.

Deferoxamine Suppresses NF- κ B Activation in Triple-Negative Breast Cancer Cells

We next investigated NF- κ B signaling to more thoroughly determine the impact of radiation and deferoxamine on cancer cell proliferation and survival (Figure 3, middle). Immunohistochemical analysis of p65 by confocal microscopy following radiation and/or deferoxamine treatment showed high cytosolic presence of p65 in comparison to nuclear presence. This finding indicates that radiation and deferoxamine prevent nuclear localization of NF- κ B and suppress activation of the NF- κ B signaling pathway, ultimately leading to a cessation of cell proliferation and cell death.

Deferoxamine Induces Apoptosis in Triple-Negative Breast Cancer Cells

Finally, we sought to determine whether deferoxamine causes cell death via the induction of apoptosis or direct necrosis (Figure 3, below). Following exposure to 25 μ M of deferoxamine, cells demonstrated a steady increase in luminescence from 0 to 24 hours (MDA-MB-231: 0 to 19371, $p=0.000$; MDA-MB-468: 0 to 8907, $p=0.010$), with no detectable increase in fluorescence. Similarly, when 10 Gy of radiation and 75 μ M of deferoxamine were administered together, both cell lines demonstrated an increase in luminescence from 0 to 24 hours (MDA-MB-231: 0 to 14061, $p=0.000$; MDA-MB-468: 0 to 6780, $p=0.001$) with no noticeable increase in fluorescence. In this assay, increased luminescence indicates the presence of phosphatidylserine in the outer leaflet of the cell membrane while increased fluorescence indicates cell membranes compromise. Thus, the time-dependent increase in luminescence that precedes any detectable increase in fluorescence, as opposed to a simultaneous increase in both luminescence and fluorescence, suggests deferoxamine is inducing apoptosis in cells and not causing direct cell necrosis.

DISCUSSION

The overall per patient complication rate following breast reconstruction is nearly 50 percent among patients who undergo radiotherapy.³⁵ While previous studies suggest deferoxamine, an FDA-approved iron chelator, is capable of alleviating the destructive effects of radiation on soft tissue and improving irradiated breast reconstruction outcomes, potential safety concerns regarding the use of deferoxamine as an angiogenic stimulator in breast cancer survivors has yet to be addressed.⁸⁻¹² In this study, we make significant progress towards defining the oncologic safety profile of deferoxamine by demonstrating that deferoxamine does not protect or enhance breast cancer viability, but rather acts cooperatively with

radiotherapy to suppress NF- κ B activation, induce apoptosis, and inhibit cancer proliferation.

We first determined that a single dose of 10 Gy of radiation is capable of significantly inhibiting triple-negative breast cancer cell proliferation. Through live cell imaging studies, we showed that cell growth was reduced just 24 hours after radiation was administered (See Figure, Supplemental Digital Content 2, which shows Live-cell imaging photos of MDA-MB-468 cells taken 24, 36, and 48 hours after no treatment (Control), 10 Gy of radiation (Radiation), and 25 μ M of deferoxamine (Deferoxamine)). These quantitative and qualitative findings corroborate the work of Zhang et al. and Zhu et al., who previously defined the dose-dependent effect of radiation on MDA-MB-231 and MDA-MB-468 cells.^{28, 30} Considering the well-established efficacy of radiation as an oncologic therapy, these results ultimately established the validity of our *in-vitro* metrics prior to investigating the impact of deferoxamine on breast cancer.

We next demonstrated that 25 μ M of deferoxamine is capable of significantly inhibiting the propagation of triple-negative breast cancer cells. A specific dose response was defined by administering deferoxamine in successively higher concentrations and quantifying the resultant change in percent cell viability and sphere number. Though less pronounced than the dose response put forth by Power Coombs et al. in 2015, we observed a significant inverse correlation between deferoxamine concentration and cell proliferation up to 100 μ M.²³ Qualitative live cell imaging studies showed substantial inhibition of cell growth just 24 hours after therapeutic administration, similar to the cellular response we observed following the administration of radiation (See Video, Supplemental Digital Content 3, which shows Live cell imaging of MDA-MB-468 cells exposed to control conditions (Control), 10 Gy of radiation (XRT), or 25 μ M of deferoxamine (DFO). Video captures cell proliferation 24 to 48 hours after therapeutic dosing. The Control well quickly reaches confluence while cells in the XRT and DFO wells struggle to proliferate. Webbed structures are healthy, attached cells while round structures are dead, lifted cells) (Video graphic 1).

Given the emerging utilization of deferoxamine to manage tissue injury secondary to radiotherapy, a complete assessment of oncologic risks must consider potential synergistic or antagonistic interactions between deferoxamine and radiotherapy. To the authors' knowledge, no previous investigations have been performed to assess for interactions between deferoxamine and radiotherapy in the context of breast cancer biology. In this study, we conservatively demonstrated that the administration of 10 Gy of radiation and 100 μ M of deferoxamine inhibits triple-negative breast cancer cell proliferation to a significantly greater degree than the administration of 10 Gy of radiation alone, suggesting a cooperative relationship between these therapies. This finding provides evidence that support the use of deferoxamine as a safely administered regenerative therapeutic for irradiated breast reconstruction.

The reduction in breast cancer cell proliferation we observed following the administration of deferoxamine either with or without radiation suggests pharmacologically induced iron chelation may disrupt key iron-dependent processes necessary for cancer proliferation. While Liu et al. reported paradoxical findings in 2016, we robustly demonstrated that

deferoxamine treatment lowers intracellular iron levels in triple-negative breast cancer cell lines.²⁷ This discrepancy is most likely attributable to a variance in concentration of deferoxamine utilized in each study (100 versus 200 μ M max dose).²⁷ Moreover, through immunohistochemical analysis of NF- κ B localization, we demonstrated that both radiation and deferoxamine suppress NF- κ B activation leading to cessation of rampant cell proliferation. Perhaps most importantly, we revealed that deferoxamine induces apoptosis in triple-negative breast cancer cells.

Despite the promising nature of these findings, there are important limitations that warrant consideration. First, cancer cell biology varies significantly, even amongst breast cancer cell lines. We thus examined two distinct ER-, PR-, and HER2- breast cancer cell lines and performed preliminary studies on MCF-7 cells to enhance the strength and applicability of our findings. Cell characteristics may also change due to unavoidable selection during the cell passaging process. To eliminate the potential for substantial modifications of cell function, we only utilized cells at or before passage ten. These *in-vitro* results justify the need for future *in-vivo* breast cancer xenograft studies to better understand whether the inhibition of cancer cell proliferation via localized iron chelation outweighs the potential increase in angiogenesis proximal to tumor formation. Indeed, deferoxamine may emerge as a potential chemotherapeutic agent to aid oncologists in the treatment of breast cancer once further and more extensive studies in areas such as pharmacogenomics have been completed. More acutely, we believe the findings of this preclinical study will begin to facilitate widespread clinical adoption of deferoxamine as a regenerative therapeutic.³⁶

CONCLUSION

In this study, we demonstrate that deferoxamine acts cooperatively with radiotherapy to inhibit triple-negative breast cancer cell proliferation. This finding is most likely attributable to the ability of deferoxamine to lower intracellular iron levels, inhibit nuclear localization of NF- κ B and suppress the NF- κ B pathway, and induce apoptosis. While this study makes significant progress towards defining the oncologic safety profile and clinical potential of deferoxamine administration for irradiated breast reconstruction, further *in-vivo* xenograft studies are warranted prior to clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The National Institutes of Health RO1 grant CA12587-06 awarded to Dr. Steven R. Buchman supported this work.

REFERENCES

1. American Cancer Society. Breast Cancer Facts & Figures 2017-2018. Atlanta: American Cancer Society, Inc 2017.
2. American Cancer Society. Cancer Treatment & Survivorship Facts & Figures 2016-2017. Atlanta: American Cancer Society, Inc 2016.

3. Early Breast Cancer Trialists' Collaborative Group (EBCTCG), Darby S, McGale P, et al. Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: Meta-analysis of individual patient data for 10,801 women in 17 randomised trials. *Lancet*. 2011;378(9804):1707–1716. [PubMed: 22019144]
4. Early Breast Cancer Trialists' Collaborative Group (EBCTCG), McGale P, Taylor C, et al. Effect of radiotherapy after mastectomy and axillary surgery on 10-year recurrence and 20-year breast cancer mortality: Meta-analysis of individual patient data for 8135 women in 22 randomised trials. *Lancet*. 2014;383(9935):2127–2135. [PubMed: 24656685]
5. Archambeau JO, Pezner R, Wasserman T. Pathophysiology of irradiated skin and breast. *Int J Radiat Oncol Biol Phys*. 1995;31(5):1171–1185. [PubMed: 7713781]
6. Cordeiro PG, Pusic AL, Disa JJ, McCormick B, VanZee K. Irradiation after immediate tissue expander/implant breast reconstruction: Outcomes, complications, aesthetic results, and satisfaction among 156 patients. *Plast Reconstr Surg* 2004;113(3):877–881. [PubMed: 15108879]
7. Propper RD, Cooper B, Rufo RR, et al. Continuous subcutaneous administration of deferoxamine in patients with iron overload. *N Engl J Med*. 1977;297(8):418–423. [PubMed: 882111]
8. Mericli AF, Das A, Best R, Rodeheaver P, Rodeheaver G, Lin KY. Deferoxamine mitigates radiation-induced hypovascularity and improves tissue elasticity in a rat irradiated TRAM flap model. *Plast Reconstr Surg*. 2014;133(3 Suppl):124. [PubMed: 25942218]
9. Mericli AF, Das A, Best R, Rodeheaver P, Rodeheaver G, Lin KY. Deferoxamine mitigates radiation-induced tissue injury in a rat irradiated TRAM flap model. *Plast Reconstr Surg*. 2015;135(1):124e–134e.
10. Duscher D, Neofytou E, Wong VW, et al. Transdermal deferoxamine prevents pressure-induced diabetic ulcers. *Proc Natl Acad Sci U S A*. 2015;112(1):94–99. [PubMed: 25535360]
11. Duscher D, Januszyk M, Maan ZN, et al. Comparison of the hydroxylase inhibitor dimethylxalylglycine and the iron chelator deferoxamine in diabetic and aged wound healing. *Plast Reconstr Surg*. 2017;139(3):695e–706e.
12. Flacco J, Chung N, Blackshear CP, et al. Deferoxamine preconditioning of irradiated tissue improves perfusion and fat graft retention. *Plast Reconstr Surg*. 2018;141(3):655–665. [PubMed: 29135894]
13. Felice PA, Nelson NS, Page EE, et al. Amifostine reduces radiation-induced complications in a murine model of expander-based breast reconstruction. *Plast Reconstr Surg*. 2014;134(4):551e–560e.
14. Polyatskaya Y, Nelson NS, Rodriguez JJ, et al. Prophylactic amifostine prevents a pathologic vascular response in a murine model of expander-based breast reconstruction. *J Plast Reconstr Aesthet Surg*. 2016;69(2):234–240. [PubMed: 26631290]
15. Rodriguez JJ, Kung T, Wang Y, et al. Changes in skin vascularity in a murine model for postmastectomy radiation. *Ann Plast Surg*. 2016;76(5):494–498. [PubMed: 26418787]
16. Ram M, Singh V, Kumawat S, et al. Deferoxamine modulates cytokines and growth factors to accelerate cutaneous wound healing in diabetic rats. *Eur J Pharmacol*. 2015;764:9–21. [PubMed: 26101070]
17. Beerepoot LV, Shima DT, Kuroki M, Yeo KT, Voest EE. Up-regulation of vascular endothelial growth factor production by iron chelators. *Cancer Res*. 1996;56(16):3747–3751. [PubMed: 8706019]
18. Toyokuni S Role of iron in carcinogenesis: Cancer as a ferrotoxic disease. *Cancer Sci*. 2009;100(1):9–16. [PubMed: 19018762]
19. Liu Y, Cui Y, Shi M, Zhang Q, Wang Q, Chen X. Deferoxamine promotes MDA-MB-231 cell migration and invasion through increased ROS-dependent HIF-1 α accumulation. *Cell Physiol Biochem*. 2014;33(4):1036–1046. [PubMed: 24732598]
20. Hann HW, Stahlhut MW, Rubin R, Maddrey WC. Antitumor effect of deferoxamine on human hepatocellular carcinoma growing in athymic nude mice. *Cancer*. 1992;70(8):2051–2056. [PubMed: 1327484]
21. Donfrancesco A, Deb G, De Sio L, Cozza R, Castellano A. Role of deferoxamine in tumor therapy. *Acta Haematol*. 1996;95(1):66–69. [PubMed: 8604589]

22. Torti SV, Torti FM. Iron and cancer: More ore to be mined. *Nat Rev Cancer*. 2013;13(5):342–355. [PubMed: 23594855]
23. Power Coombs MR, Grant T, Greenshields AL, Arsenault DJ, Holbein BE, Hoskin DW. Inhibitory effect of iron withdrawal by chelation on the growth of human and murine mammary carcinoma and fibrosarcoma cells. *Exp Mol Pathol*. 2015;99(2):262–270. [PubMed: 26210486]
24. Jiang XP, Elliott RL. Decreased iron in cancer cells and their microenvironment improves cytotoxicity of breast cancer cells by natural killer cells. *Anticancer Res*. 2017;37(5):2297–2305. [PubMed: 28476795]
25. Wang G, Shen G, Yin T. In vitro assessment of deferoxamine on mesenchymal stromal cells from tumor and bone marrow. *Environ Toxicol Pharmacol*. 2017;49:58–64. [PubMed: 27915123]
26. Rakha EA, El-Sayed ME, Green AR, Lee AH, Robertson JF, Ellis IO. Prognostic markers in triple-negative breast cancer. *Cancer*. 2007;109(1):25–32. [PubMed: 17146782]
27. Liu P, He K, Song H, Ma Z, Yin W, Xu LX. Deferoxamine-induced increase in the intracellular iron levels in highly aggressive breast cancer cells leads to increases cell migration by enhancing TNF- α -dependent NF- κ B signaling and TGF- β signaling. *J Inorg biochem*. 2016;160:40–48. [PubMed: 27138103]
28. Zhang Z, Wang H, Prasad G, et al. Radiosensitization by antisense anti-MDM2 mixed-backbone oligonucleotide in in vitro and in vivo human cancer models. *Clin Cancer Res*. 2004;10(4):1263–1273. [PubMed: 14977824]
29. Lautenschlaeger T, Perry J, Peereboom D, et al. In vitro study of combined cilengitide and radiation treatment in breast cancer cell lines. *Radiat Oncol*. 2013;8:1–10. [PubMed: 23280007]
30. Zhu R, Li W, Xu Y, Wan J, Zhang Z. Upregulation of BTG1 enhances the radiation sensitivity of human breast cancer in vitro and in vivo. *Oncol Rep*. 2015;34(6):3017–3024. [PubMed: 26503430]
31. Voest EE, Rooth H, Neijt JP, van Asbeck BS, Marx JJ. The in vitro response of human tumour cells to desferrioxamine is growth medium dependent. *Cell Prolif*. 1993;26(1):77–88. [PubMed: 8439591]
32. Bajbouj K, Shafarin J, Hamad M. High-dose deferoxamine treatment disrupts intracellular iron homeostasis, reduces growth, and induces apoptosis in metastatic and nonmetastatic breast cancer cell lines. *Technol Cancer Res Treat*. 2018;17:1–11.
33. Donneys A, Weiss DM, Deshpande SS, et al. Localized deferoxamine injection augments vascularity and improves bony union in pathologic fracture healing after radiotherapy. *Bone*. 2013;52(1):318–325. [PubMed: 23085084]
34. Johnson S, Chen H, Lo PK. In vitro tumorsphere assays. *Bio Protoc*. 2013;3(3):e325.
35. Muresan H, Lam G, Cooper BT, et al. Impact of evolving radiation therapy techniques on implant-based breast reconstruction. *Plast Reconstr Surg*. 2017;139(6):1232e–1239e.
36. Momeni A, Rapp S, Donneys A, Buchman SR, Wan DC. Clinical use of deferoxamine in distraction osteogenesis of irradiated bone. *J Craniofac Surg*. 2016;27(4):880–882. [PubMed: 27171947]

Figure 1:

Percent cell viability and sphere number analysis of FF, MDA-MB-231, and MDA-MB-468 cells following exposure to (Above) 0, 5, or 10 Gy of radiation or (Below) 0, 25, 50, 75, or 100 μM of deferoxamine. Statistical significance relative to 0 Gy or 0 μM was determined via one-way ANOVA (* $p < 0.05$, ** $p < 0.01$). Error bars represent standard error.

Figure 2:

(Above) Percent cell viability and sphere number analysis of FF, MDA-MB-231, and MDA-MB-468 cells following administration of 10 Gy of radiation and 0, 25, 50, 75, or 100 μM of deferoxamine. Statistical significance relative to 10 Gy plus 0 μM was determined via one-way ANOVA (* $p < 0.05$, ** $p < 0.01$). Error bars represent standard error. (Below) Fluorescent images of tdTomato-tagged MDA-MD-468 cells 72 hours after therapeutic dosing reveal control cells are confluent while cells administered radiation (10 Gy) and/or deferoxamine (100 μM) are sparse. Tumorsphere images depict large circular colonies in the control group and several fragmented bunches following therapeutic dosing.

Figure 3:

(Above) Intracellular iron concentration of MDA-MB-231 and MDA-MB-468 cells following deferoxamine administration and accompanying data. Error represents standard deviation. (middle) Immunohistochemical analysis of NF- κ B localization in MDA-MB-231 cells. Radiation and/or deferoxamine treatment inhibits nuclear localization and suppresses activation. DAPI was used to stain nuclei. (below) Change in luminescence in MDA-MB-231 and MDA-MB-468 cells following deferoxamine administration in the absence (25 μ M) and presence (75 μ M) of radiation. The increase in luminescence during the first 24 hours of drug exposure suggests the induction of apoptosis. Fluorescence readings remained negligible throughout. Errors bars represent standard error.