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## Sildenafil Protects Endothelial Cells From Radiation-Induced Oxidative Stress

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## Abstract

**Introduction:** The etiology of radiation-induced erectile dysfunction (ED) is complex and multifactorial, and it appears to be mainly atherogenic.

**Aim:** To focus on vascular aspects of radiation-induced ED and to elucidate whether the protective effects of sildenafil are mediated by attenuation of oxidative stress and apoptosis in the endothelial cells.

**Methods:** Bovine aortic endothelial cells (BAECs), with or without pretreatment of sildenafil (5  $\mu$ M at 5 minutes before radiation), were used to test endothelial dysfunction in response to external beam radiation at 10e15 Gy. Generation of reactive oxygen species (ROS) was studied. Extracellular hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was measured using the Amplex Red assay and intracellular H<sub>2</sub>O<sub>2</sub> using a fluorescent sensor. In addition, ROS superoxide (O<sub>2</sub><sup>•-</sup>) was measured using a O<sub>2</sub><sup>•-</sup>- chemiluminescence enhancer. Both H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup> are known to reduce the bioavailability of nitric oxide, which is the most significant chemical mediator of penile erection. Generation of cellular peroxynitrite (ONOO<sup>-</sup>) was measured using a chemiluminescence assay with the PNCL probe. Subsequently, we measured the activation of acid sphingomyelinase (ASMase) enzyme by radioenzymatic assay using [<sup>14</sup>C-methylcholine] sphingomyelin as substrate, and the generation of the proapoptotic C<sub>16</sub>-ceramide was assessed using the diacylglycerol kinase assay. Endothelial cells apoptosis was measured as a readout of these cells' dysfunction.

**Main Outcome Measures:** Single high-dose radiation therapy induced NADPH oxidases (NOXs) activation and ROS generation via the proapoptotic ASMase/ceramide pathway. The radio-protective effect of sildenafil on BAECs was due to inhibition of this pathway.

**Results:** Here, we demonstrate for the first time that radiation activated NOXs and induced generation of ROS in BAECs. In addition, we showed that sildenafil significantly reduced radiation-induced O<sub>2</sub><sup>•-</sup> and as a result there was reduction in the generation of peroxynitrite in these cells. Subsequently, sildenafil protected the endothelial cells from radiation therapy-induced apoptosis.

**Strengths and Limitations:** This is the first study demonstrating that single high-dose radiation therapy induced NOXs activation, resulting in the generation of O<sub>2</sub><sup>•-</sup> and peroxynitrite in endothelial cells. Sildenafil reduced ROS generation by inhibiting the ASMase/ceramide pathway. These studies should be followed in an animal model of ED.

**Conclusions:** This study demonstrated that sildenafil protects BAECs from radiation-induced oxidative stress by reducing NOX-induced ROS generation, thus resulting in decreased endothelial dysfunction. Therefore, it provides a potential mechanism to better understand the atherogenic etiology of postradiation ED.

## Keywords

Erectile Dysfunction; Endothelial Damage; Radiation; Reactive Oxygen Species; Oxidative Stress

## INTRODUCTION

Patients with localized prostate cancer treated with external beam radiotherapy frequently develop erectile dysfunction (ED),<sup>1</sup> which can adversely affect quality of life among patients and their partners.<sup>2</sup> The etiology of ED following radiotherapy is multifactorial and includes psychological, neurologic, and vascular disruptions.<sup>3</sup> Zelefsky et al<sup>4</sup> analyzed penile Doppler ultrasonography in patients with postradiation ED to elucidate its etiology and demonstrated that the majority of patients had abnormalities suggestive of atherogenic ED. Novel techniques such as intensity-modulated radiotherapy with image guidance have increased the precision of treatment delivery and enabled reductions in safety margins that are used to account for delineation uncertainties and prostate motion during treatment.<sup>5-7</sup> Because safety margins of 3e10 mm are generally still applied in external beam radiotherapy, vascular damage inevitably occurs to some extent since the neurovascular bundle and relevant penile blood vessels are always exposed to some radiation dose due to their close proximity to the prostate.<sup>8</sup>

Specifically, endothelial cells of small arteries, capillaries, and sinusoids are highly radiosensitive and therefore frequently affected by ionizing radiation.<sup>9</sup> Redox signaling has emerged as an essential mechanism in the regulation of the biological activity of a variety of cells. Overwhelming evidence has accumulated showing that in vascular cells, non-mitochondrial NADPH oxidase (NOX) is a major source of  $O_2^{\bullet-}$  in the vessel wall for the redox regulation of vascular endothelial and smooth muscle function.<sup>10-17</sup> The non-mitochondrial NOX-derived  $O_2^{\bullet-}$  constitutes greater than 95% of  $O_2^{\bullet-}$  production in the vasculature, especially upon stimulation.<sup>14,18</sup> Recently, it has been demonstrated that the phosphorylation and translocation of NOX subunits are assembled together within the ceramide-rich platforms (CRMs) generated in arterial endothelial cells in response to Fas ligand, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), endostatin, and homocysteine.<sup>19-26</sup>

NOXs, which are expressed in endothelial cells, play an important role in the development of vascular dysfunction.<sup>27,28</sup> One of the initial steps in NOX-2 activation is the translocation of its subunit p47<sup>phox</sup> from the cytoplasm to the plasma membrane.<sup>29</sup> The primary function of NOXs is to produce reactive oxygen species (ROS), which under normal conditions are involved in cell signaling and homeostasis.<sup>27,28</sup> In response to stimuli such as ionizing radiation, NOX activity is markedly increased and high levels of ROS are produced.<sup>30</sup> Overproduction of ROS in turn induces oxidative stress, which leads to endothelial dysfunction. Endothelial dysfunction is a known contributing factor in cardiovascular diseases and ED.<sup>31</sup>

The primary treatment of radiation-induced ED consists of phosphodiesterase-5 inhibitors (PDE-5is).<sup>32,33</sup> Zelefsky et al<sup>34</sup> conducted a randomized clinical trial to determine whether the PDE-5i, sildenafil (Viagra; Pfizer, New York, NY, USA), could be used to preserve erectile function in patients with prostate cancer with adequate baseline erectile function undergoing radiotherapy. Daily treatment with sildenafil (50 mg) during radiotherapy, and up to 6 months after completion, was associated with improved erectile function compared with placebo; effects were maintained well after termination of treatment.<sup>34</sup> Previous studies

by Gebaska et al<sup>35</sup> have demonstrated PDE-5 activity in human, mouse, and bovine aortic endothelial cells (BAECs) within the CRMs. In this study, we only focused on the vascular aspects of postradiotherapy ED in general and studied endothelial cell function specifically. The objective of our study was to elucidate the cellular mechanism by which PDE-5i, sildenafil, attenuates endothelial dysfunction and protects against radiation-induced ED. We showed that sildenafil inhibited radiation-induced NOX generation of ROS in BAEC and protected them from apoptotic death via the acid sphingomyelinase (ASMase)/ceramide pathway.

## MATERIALS AND METHODS

### Cell Cultures

Cloned populations of BAECs were established from the intima of bovine aorta as previously described.<sup>36,37</sup> BAECs were favored over human umbilical vein endothelial cells, as these cells generate tighter monolayers with nearly 100% of the cells in G<sub>0</sub>/G<sub>1</sub>. In addition, in vitro studies using BAECs generally translate well in animal and clinical studies. Cultures were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with glucose (1 g/L), bovine calf serum (5%), penicillin (100 U/mL), streptomycin (100 µg/L), and L-glutamine (2 mM) at 37°C in a humidified 10% CO<sub>2</sub> chamber. For experiments, confluent monolayers of BAECs were incubated overnight in serum-free DMEM supplemented with 0.2% human albumin unless otherwise stated. All experiments as described to follow were performed in triplicate.

### Radiotherapy Technique

For all experiments, cells were irradiated using a Shephard Mark I irradiator containing a <sup>137</sup>Cs source. Irradiation was carried out at a rate of 1.66 Gy/min. The radiotherapy dose used varied between experiments and is reported for each experiment in the Results section depending on the assay used and the biological readout.

### Quantification of Endothelial Apoptosis

As previously described,<sup>37</sup> the DNA-binding fluorochrome *bis*-benzimidazole trihydrochloride (Hoechst-33258; Sigma-Aldrich, St. Louis, MO, USA) was used for detection of morphologic changes in nuclear chromatin of BAECs undergoing apoptosis. These first experiments on quantification of endothelial apoptosis were used to determine the optimal sildenafil dose (1, 5, or 10 µM), incubation time (5, 30, or 60 minutes), and radiation dose (10, 15, or 20 Gy). After pretreatment with sildenafil or placebo, cultured cells were irradiated. Eight hours following irradiation, BAEC were fixed with paraformaldehyde (10%), washed with phosphate-buffered saline (PBS), and stained with 50 µL of *bis*-benzimidazole trihydrochloride (Hoechst-33258) for 10 minutes. Quantification of apoptosis was done using a Zeiss Axio Imager Z-2 (Carl Zeiss AS, Oslo, Norway) with a fluorescent filter allowing DAPI imaging. A total of 400 cells were counted, blinded, in triplicates, for each data point.

## ROS Measurement

**ROS: 2',7'-Dichlorofluorescein Diacetate Detection Assay (DCF-DA)**—General cellular ROS production was assessed using DCF-DA (Abcam, Cambridge, United Kingdom). DCF-DA is a nonfluorescent compound that is membrane permeable and becomes highly fluorescent upon oxidation by ROS. BAECs were seeded in a clear-bottom 96-well plate at a concentration of  $4\text{--}6 \times 10^4$  cells/well and allowed to attach overnight. On the day of the experiment, cells were washed once with PBS and stained with  $25 \mu\text{M}$  DCF-DA in buffer and incubated for 45 minutes at  $37^\circ\text{C}$ . Cells were washed once with PBS and  $100 \mu\text{L}$  of phenol-free media was added with treatment. Sildenafil  $5 \mu\text{M}$  or placebo was added 5 minutes before irradiation. Cells were irradiated with 10 Gy and the fluorescent signal was read at excitation of 485 nm and at emission of 535 nm. tert-Butyl hydrogen peroxide (Sigma) was used as positive control according to the manufacturer's instructions.

**ROS: Superoxide**—The Diogenes System (National Diagnostics, Atlanta, GA, USA) is a superoxide ( $\text{O}_2^{\bullet-}$ ) chemiluminescence enhancer that was used to measure  $\text{O}_2^{\bullet-}$  production. BAECs were seeded in a flat white-bottom 96-well plate at a concentration of  $4\text{--}6 \times 10^4$  cells per well. Cells were allowed to attach overnight. One day before the experiment, cells were washed once with PBS and media was changed overnight to phenol-free media including penicillin ( $100 \text{ U/mL}$ ), streptomycin ( $100 \mu\text{g/L}$ ), and L-glutamine ( $2 \text{ mM}$ ). On the day of the experiment, cells were treated with  $100 \mu\text{L}$  of Diogenes Complete Enhancer Solution (National Diagnostics) and either sildenafil ( $5 \mu\text{M}$ ) or placebo was added. Cells were subsequently irradiated with 10 Gy and the light output was read on a luminescent microplate reader (Infinite M1000 Pro; Tecan Group, Männedorf, Switzerland) for up to 20 minutes. Xanthine ( $100 \mu\text{L}$  of  $0.5 \text{ mM}$  in  $50 \text{ mM}$  potassium phosphate buffer)/xanthine oxidase ( $20 \mu\text{L}$  of  $10^{-3}$  units per mL in  $50 \text{ mM}$  potassium phosphate buffer) solution was added as a positive control according to the manufacturer's instructions.

**ROS: Hydrogen Peroxide**—The Amplex Red assay (Molecular Probes, Eugene, OR, USA) was used to measure extracellular hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), which can be generated by radiation directly. BAECs were seeded in 6-well plates and cultured until confluent. Medium was changed overnight to  $1 \text{ mL}$  of phenol-free DMEM with  $0.2\%$  human albumin. Cells were incubated with Amplex Red ( $40 \mu\text{M}$ ) and horseradish peroxidase ( $20 \text{ mU/mL}$ ). BAECs were pretreated with sildenafil  $5 \mu\text{M}$  or placebo for 5 minutes and subsequently irradiated with 10 Gy and incubated at  $37^\circ\text{C}$  for 30 minutes. In the presence of peroxidase, secreted  $\text{H}_2\text{O}_2$  reacts with Amplex Red, producing the red-fluorescent resorufin. A fluorescence microplate reader (Infinite M1000 Pro; Tecan) was used to measure fluorescence at excitation and emission maxima of  $571/585 \text{ nm}$ .

Changes in intracellular  $\text{H}_2\text{O}_2$  levels, which might be generated by radiation-induced NOX, were measured using the  $\text{H}_2\text{O}_2$  sensor HyPer (Evrogen, Moscow, Russia).<sup>38</sup> HyPer is a genetically encoded fluorescent sensor capable of detecting intracellular  $\text{H}_2\text{O}_2$  produced by living cells. In absence of  $\text{H}_2\text{O}_2$ , HyPer has 2 excitation peaks with maximal excitation at  $420 \text{ nm}$  and  $500 \text{ nm}$ , and an emission maximum at  $516 \text{ nm}$ . Upon exposure to  $\text{H}_2\text{O}_2$ , a proportional shift in the excitation peak at  $420 \text{ nm}$  to  $500 \text{ nm}$  occurs, allowing ratiometric measurements of intracellular  $\text{H}_2\text{O}_2$ . For these experiments, BAECs were seeded in a 4-

chamber well slide (Lab-Tek) at a concentration of  $4\text{--}6 \times 10^4$  and allowed to attach overnight 1 day before transfection. Cells were transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and within the recommended range of ratio between reagent and DNA. Two days after transfection, media were changed overnight to serum-free DMEM with 0.2% human albumin. Before irradiation, 5 transfected single cells were identified per chamber using a ZEN Microscope System (Carl Zeiss, Oberkochen, Germany). Periodic images of the transfected cells were first taken at baseline, and the cells were subsequently irradiated with 10 Gy. After irradiation, the cells were re-identified and imaging was continued for up to 30 minutes.  $\text{H}_2\text{O}_2$  was added at this point as a positive control in separate wells.

**ROS: Peroxynitrite**—Peroxynitrite was measured with a chemiluminescence assay using the probe PNCL.<sup>39</sup> In brief, peroxynitrite reacts selectively with the probe, PNCL, to initiate a chemiluminescent reaction that can be measured using a luminescence plate reader. BAECs were plated on white opaque 24-well plates and cultured until cells reached confluence. Probe was added 30 minutes before irradiation and sildenafil ( $5 \mu\text{M}$ ) was added 5 minutes before irradiation. Cells were irradiated with 10 Gy and luminescence was measured 10 minutes after irradiation using the Cytation 5 imager (BioTek Instruments, Winooski, VT, USA). Signal of probe alone in media with no cells was subtracted from all readings as background signal. Activity of the probe under irradiation was examined before the experiment using SIN-1 as positive control and was found to be unaffected.

### ASMase Activity

ASMase activity in BAECs was quantified by radioenzymatic assay using [ $^{14}\text{C}$ -methylcholine] sphingomyelin (Amersham Biosciences Corp., Piscataway, NJ, USA) as substrate.<sup>40</sup> To summarize in brief, BAECs were first preincubated for 5 minutes with  $5 \mu\text{M}$  sildenafil or placebo. Cells were then irradiated to 10 Gy on ice and subsequently incubated at  $37^\circ\text{C}$ . Following incubation, cells were placed on ice. The conditioned media were collected, and cells were washed with cold PBS. Cells were lysed in PBS containing 0.2% Triton X-100 and collected. The lysate protein concentration was measured using the bicinchoninic acid assay. For assaying the activity, cell lysate was incubated with [ $^{14}\text{C}$ -methylcholine]-sphingomyelin substrate (0.026 mCi/9.5 nmol) in 250 mM sodium acetate, pH 5.0 supplemented with 1 mM EDTA, and 0.1% Triton X-100. After 2 hours, reactions were terminated with chloroform: methanol (2:1) and ASMase activity was quantified using a Beckman Packard 2200 CA Tricarb scintillation counter.

### Ceramide Quantification

Ceramide levels were quantified as previously described.<sup>41</sup> Cells were preincubated for 5 minutes with  $5 \mu\text{M}$  sildenafil or placebo and subsequently irradiated to 10 Gy. At predefined time points after irradiation, cells were washed with ice-cold PBS and placed on ice. Cells were detached with a scraper and lipids were extracted to equal volumes of methanol and chloroform, and 0.6 volume of buffered saline solution (135 mM NaCl, 4.5 mM KCl, 1.5 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgCl}_2$ , 5.6 mM glucose, 10 mM HEPES [pH 7.2], 10 mM EDTA). The diacylglycerol kinase assay was used to quantify ceramide levels.

## Confocal Microscopy

BAECs were seeded in 8-well chamber slides (Lab-Tek) at  $4 \times 10^4$  cells/chamber and cultured overnight until 70–80% confluence. Cells were irradiated to 15 Gy and at 1 minute and 5 minutes; afterwards, the cells were fixed with 4% paraformaldehyde in PBS pH 7.4 for 10 minutes at room temperature.

For ceramide staining, cells were washed 3 times with 0.1% Tween-20 in PBS (PBST) and subsequently blocked with 5% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 minutes at room temperature, then incubated with mouse-anti-ceramide antibody (MID 15B4 IgM, 1:50 dilution; Enzo Life Sciences, Inc., Farmingdale, NY, USA) for 1 hour at room temperature. Following with 3 washes with PBST, the slides were incubated with Cy3-conjugated donkey anti-mouse IgM (1:300; Jackson ImmunoResearch Laboratories).

For p47<sup>phox</sup> staining, the slides were washed 3 times with PBST, permeabilized with 0.1% Triton X100 for 1 minute at room temperature, and blocked with 10% goat serum (MP Biomedicals, Santa Ana, CA, USA). Subsequently, slides were incubated with the p47<sup>phox</sup> primary antibody (p47-phox D-10 IgG, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 hour at room temperature. Following 3 washes, the slides were incubated with goat anti-mouse IgG (DyLight 488 conjugated, 1:200; Thermo Fisher Scientific, Rockford, IL, USA) for 1 hour at room temperature. Finally, the slides were counter stained with 1  $\mu\text{g}/\text{mL}$  DAPI for 1 minute and mounted with coverslips. The images were taken with a microscope at 40 $\times$  magnification.

## Statistical Analysis

Data are presented as mean  $\pm$  standard error. SPSS version 21 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 6.0 (La Jolla, CA, USA) were used to analyze and plot the data. Differences between the 2 groups were analyzed using the independent *t* test, whereas the analysis of variance test was used to compare multiple groups. The Bonferroni method of correction was applied for multiple comparisons.

## RESULTS

### Radiation-Induced NOX Activation and Generation of ROS

We have previously shown that single-dose radiotherapy (SDRT) induces microvascular dysfunction via activation of the ASMase/ceramide pathway.<sup>41,42</sup> ASMase activation triggers generation of CRMs, NOX activation, and subsequent production of ROS, resulting in microvascular dysfunction.<sup>43</sup> In this work, we demonstrated NOX activation in response to SDRT in BAEC (Figure 1). Using confocal microscopy, we showed that SDRT-induced co-localization of ceramide with the p47<sup>phox</sup>, NOX subunit, as early as 1 minute post-SDRT, which decreased at 5 minutes post-SDRT. These results demonstrate that SDRT induces NOX activation via ASMase/ceramide pathway and CRMs assembly, as previously demonstrated by our collaborators in these cells in response to Fas ligand and TNF- $\alpha$ .<sup>26</sup>

Next, we evaluated whether sildenafil protected BAECs from SDRT-induced apoptosis. Figure 2 represents the results of experiments that were conducted to determine the optimal sildenafil treatment dose, radiation dose, and timing for all subsequent experiments. First, sildenafil (5  $\mu\text{M}$ ) added 5 minutes before 10 Gy radiation inhibited SDRT-induced apoptosis at 8 hours by 45% from 17.4% (SDRT alone) to 9.6% (SDRT + sildenafil 5  $\mu\text{M}$ ) (Figure 2A). At a greater dose (10  $\mu\text{M}$ ), sildenafil was toxic, inducing 12.2% apoptosis in BAECs by itself, and 24.6% apoptosis in combination with 10 Gy. All subsequent experiments were therefore conducted using sildenafil at the dose of 5  $\mu\text{M}$ . A time—course experiment showed that the effects of sildenafil on SDRT-induced apoptosis were not significantly improved if preincubation times were longer than 5 minutes (Figure 2B). Therefore, we continued these studies using 5 minutes of preincubation with sildenafil before SDRT. In a radiation—dose escalation experiment, the protective effect of sildenafil was most profound at 10 Gy (17.6% apoptotic cells for SDRT alone vs 8.4% for sildenafil-treated cells, yielding 43% inhibition) and also present at doses up to 20 Gy (25.6% apoptosis vs 16.7%, a 35% reduction) (Figure 2C). On the basis of these results, further experiments were conducted using sildenafil doses of 5  $\mu\text{M}$ , preincubated at 5 minutes before irradiation of 10 Gy, whereas greater radiation doses were optional as similar effects were found.

A recent study published from our group demonstrated that SDRT-induced apoptosis in BAEC was prevented by ROS scavengers.<sup>30</sup> Therefore, we tested whether diphenyleneiodonium (DPI), a known inhibitor of several NOXs, responsible for intracellular ROS production,<sup>44</sup> would protect BAECs from radiation-induced apoptosis. DPI (10  $\mu\text{M}$ ), added 30 minutes before 10 Gy treatment, reduced SDRT-induced apoptosis by 79% as compared with 10-Gy treatment alone, from 12.8% to 2.7% (Figure 2D). Similar results were found in these cells in response to 15 Gy. These data corroborate that intracellular NOX-derived ROS play a significant role in SDRT-induced endothelial apoptotic cell death.

### Effects of Sildenafil on Radiation-Induced ROS in Endothelial Cells

We further investigated whether the protective effect of sildenafil on BAECs was mediated by means of ROS inhibition. First, we analyzed the type of ROS generated in response to SDRT in BAECs. BAECs were stained with the DCF-DA and incubated for 45 minutes. Subsequently, the DCF-DA assay was used to measure general cellular ROS generation in BAECs after exposure to 10 Gy. ROS generation increased significantly 2 minutes after exposure to 10 Gy and gradually decreased from 30 minutes post-SDRT onwards. ROS generation immediately after radiation is largely the result of the direct reaction between ionizing radiation and  $\text{H}_2\text{O}$ , which produces  $\text{H}_2\text{O}_2$  (data not shown).<sup>45</sup> Subsequent to ASMase activation, ceramide generation, and the assembly of the CRMs there is a second wave of ROS generation due to NOX activation within the CRMs.<sup>28,29,43,46–48</sup> Sildenafil (5  $\mu\text{M}$ ) was added to BAECs 5 minutes before exposure to 10 Gy. A 24% inhibition of the second wave of ROS production was observed at 30 minutes post-SDRT (Figure 3).

Next, we identified and quantified different species of ROS generated by NOX in endothelial cells, in particular superoxide ( $\text{O}_2^{\bullet-}$ ) and  $\text{H}_2\text{O}_2$ , which have emerged as potent inducers of oxidative stress and microvascular dysfunction. Irradiation with 10 Gy induced a substantial



increase in  $O_2^{\bullet-}$  activity in BAEC at 2 minutes after irradiation, which rapidly decayed to almost baseline by 5 minutes (Figure 4). Pretreatment with either of sildenafil (5  $\mu M$ ; 5 minutes), or DPI (10  $\mu M$ ; 30 minutes), inhibited SDRT-induced  $O_2^{\bullet-}$  production at 2 minutes after treatment by 27% and 29%, respectively. These results suggest that NOXs were the source for the generation of this intracellular  $O_2^{\bullet-}$  and that sildenafil was able to inhibit NOXs-induced  $O_2^{\bullet-}$  activity detected in these cells.

Radiation induced a significant increase of extracellular  $H_2O_2$  levels in BAECs, which was not affected by pretreatment with sildenafil (5  $\mu M$ ), as expected (Figure 5A), since this is generated due to the radiation interaction with the extracellular  $H_2O$ . Similarly, sildenafil had no effect on the radiation-induced intracellular  $H_2O_2$  levels in these cells (Figure 5B), which fits with the proposed model in Figure 3 (adopted from Zhang et al<sup>25</sup>). Using the Amplex Red assay, we showed that at 2 minutes after 10 Gy,  $H_2O_2$  fluorescence intensity did not significantly decrease with pretreatment with 5  $\mu M$  sildenafil (Figure 5B). In contrast, pretreatment of BAEC with DPI (10  $\mu M$ ) 30 minutes before radiation had a significant inhibitory effect on the intracellular  $H_2O_2$  levels (13% increase over baseline vs 26% for untreated cells,  $P < .05$ ; Figure 5B). These results indicate that the effects of sildenafil are most likely not mediated through inhibition of  $H_2O_2$  production in these cells.

Finally, we measured intracellular peroxynitrite levels at baseline and in response to 10 Gy with and without sildenafil pretreatment. A single dose of 10 Gy significantly increased the levels of intracellular peroxynitrite. However, in cells pretreated with 5  $\mu M$  sildenafil for 5 minutes, there was more than a 50% inhibition in the peroxynitrite generation (Figure 5C), resulting from sildenafil inhibition of NOXs-induced  $O_2^{\bullet-}$  generation in these cells.

### Effect of Sildenafil on Radiation Activation of ASMase/Ceramide Pathway

Vascular endothelial cells express 20-fold increased levels of secretory ASMase than any other cell in the body,<sup>49</sup> an enzyme responsible for generation of ceramide. ASMase activity increased 2 minutes after irradiation and peaked at 5 minutes in BAEC, similar to our previous results in these cells in response to this radiation dose. Preincubation for 5 minutes with 5  $\mu M$  sildenafil inhibited ASMase activity by 83% at 5 minutes post-SDRT (0.7 pmols/ $\mu g/h$  for sildenafil vs 4.3 pmols/ $\mu g/h$  for controls) (Figure 6A), resulting in a subsequent 42% inhibition in ceramide levels in these cells as compared with the untreated, irradiated cells (1,881.6 pmol/ $10^6$  cells vs 3,258.9 pmol/ $10^6$  cells) (Figure 6B). These results indicate that sildenafil abrogated ASMase activity either directly or indirectly in endothelial cells.

## DISCUSSION

Pelvic radiation therapy, in particular, that was used for prostate cancer is well recognized to be associated with long-term ED.<sup>50</sup> The etiology of postradiotherapy ED is complex and includes nerve, vascular, and psychological disruptions.<sup>3</sup> Although neural injury occurs with radiation also, the degree to which it is a contributor to ED is unclear, as many men continue to respond to PDE-5i many years after radiation monotherapy.<sup>51</sup> In the current study, we focused on vascular causes of ED only, since the literature indicates that much of the means by which radiation induced-ED in the latter population is through vascular damage, indeed, endarteritis obliterans. Endothelial cells lining the penile arteries and the sinusoids of the

corpora cavernosa are sensitive to radiation and are directly damaged in a dose- and time-dependent manner. Endothelial cell damage and microvessel rupture lead to luminal stenosis and arterial insufficiency that can arise months to years after radiation exposure. Blood capillaries and sinusoids are considered especially radiosensitive, since endothelium comprises the major portion of their walls. Pathologic analysis of these vessels has demonstrated characteristic findings on light microscopy: vessel dilation, luminal irregularities and asymmetry, and thrombosis.<sup>9</sup> The net effect of damage to the microvascular network of vessels is ischemia. Small-sized arteries (up to 100  $\mu\text{m}$  in external diameter) also undergo thrombosis in the acute or delayed phase after irradiation.<sup>9</sup> Pathologic analysis of these vessels reveals fibrosis, hyalinization, and build-up of lipid-laden macrophages. Medium-sized (100–500  $\mu\text{m}$ ) and large-sized (>500  $\mu\text{m}$ ) are less radiosensitive than smaller arteries. Pathologic sequelae of irradiation develop at a relatively delayed time point and are characterized by collagen and fibroblast deposition, resulting in luminal narrowing and obstruction.<sup>9</sup>

Few clinical studies have focused on the vascular etiology of ED in patients after prostate radiation. In a hemodynamic analysis of such men, Mulhall et al<sup>52</sup> reported on patients who underwent cavernosometry, and 100% were shown to have cavernosal artery insufficiency and 90% corporal veno occlusive dysfunction. In one half of the patients with CVOD, leak was localized to the crura alone. These data support the data of Zelefsky and Eid<sup>4</sup> in 38 men after prostate radiation, showing that 63% had arterial insufficiency on duplex Doppler ultrasound and 32% had venous leak. In an effort to reduce ED rates after prostate radiation, Zelefsky et al<sup>11</sup> conducted a randomized trial studying the effects of the known endothelial protectant sildenafil citrate. Sildenafil 50 mg or placebo was administered daily in 279 patients during and after radiotherapy for prostate cancer. Treatment was started 3 days before start of radiotherapy and continued for 6 months. Patients were monitored using the International Index of Erectile Function questionnaire. At 24 months after radiotherapy, the erectile function domain and sexual satisfaction scores in the sildenafil group were significantly better when compared with the placebo group.

Bruner and Pisansky conducted another randomized controlled trial, which was unresponsive of PDE-5i rehabilitation after radiotherapy (RT).<sup>53</sup> The authors randomized patients to sildenafil vs placebo. The study, however, was extremely limited in 2 major ways. First, it was a very short-term analysis with patient assessment less than 12 months after study completion. Given that the effect of RT on erectile function (EF) is delayed and may take longer than 3 years to develop and usually does not start within the first 12 months, the data of Watkins Bruner et al are difficult to interpret. Second, the metric for EF preservation that was used in this study is fraught with a serious problem. Specifically, question 1 of the International Index of EF-6 was used, which asks about any erection (not one capable of penetration). This is a clinically meaningless end-point and further-more, no ED study after radical prostatectomy or RT, has ever used such an end-point. Based on the only 2 studies, we cannot be definitive in recommending PDE-5i as rehabilitation post-RT. Furthermore, the impact of timing of commencement of rehabilitation using PDE-5i in this population is impossible to define at this time.

To support the findings from the randomized controlled trial of Zelefsky et al,<sup>11</sup> in the present study, we present data suggesting that sildenafil pretreatment of irradiated endothelial cells reduces apoptosis rates and defines a potential mechanism by which this occurs. We have demonstrated that sildenafil protected endothelial cells from oxidative stress via reduction of NOX-mediated ROS formation and inhibited the proapoptotic ASMase/ceramide pathway (Figure 7).

We have shown that radiation induces NOX-mediated generation of ROS in BAECs. Others also have shown NOX activation and oxidative stress in corpora cavernosa after single-fraction RT in a rat model.<sup>54</sup> It has been previously shown that ASMase activation triggers generation of CRMs, NOX activation, and subsequent production of ROS, resulting in microvascular dysfunction.<sup>43</sup> Furthermore, it has been demonstrated by our collaborators that ASMase activation, ceramide generation, and the assembly of the CRMs in BAECs brings together the NOX subunits, thus activating the NOX enzymes in these cells, in response to FAS and TNF- $\alpha$ .<sup>26</sup> Here, we showed that SDRT induced co-localization of ceramide with p47<sup>phox</sup>, a NOX subunit, as early as 1 minute post-SDRT in BAEC. It is this SDRT-induced NOX activation and subsequent production of a second wave of ROS intracellularly that results in microvascular dysfunction (Figure 7) similar to the responses to FAS and TNF- $\alpha$  reported in these cells.

Moreover, sildenafil significantly inhibited ROS generation in general and specifically the immediate O<sub>2</sub><sup>•-</sup> generation. Endothelial cells express 4 ROS-generating NOX isoforms (eg, NOX1, NOX2, NOX4, and NOX5).<sup>29</sup> Of these, NOX1, NOX2, and NOX5 promote endothelial dysfunction, inflammation, and apoptosis.<sup>29</sup> NOX-derived O<sub>2</sub><sup>•-</sup> is a major mechanism of vascular dysfunction as this particular ROS reacts with nitric oxide (NO) to form peroxynitrite (ONOO<sup>-</sup>).<sup>29</sup> ONOO<sup>-</sup> is a potent oxidant that can disrupt crucial cell signaling pathways and initiate cell death by causing damage to macromolecules, including proteins, lipids, and more critically DNA.<sup>29,30</sup> In addition, the reaction reduces the bioavailability of NO, which leads to a loss of its vasoprotective properties, resulting in ischemia.<sup>29</sup>

In addition, we have shown here for the first time that sildenafil inhibits the proapoptotic ASMase/ceramide pathway. In a recent report from our group, Mizrachi et al demonstrated that activation of the ASMase/ceramide pathway was dependent on ROS formation for induction of endothelial dysfunction in the radiation-induced microvascular injury, which resulted in the salivary gland hypofunction.<sup>30</sup> In that study, BAECs were preincubated with ROS scavengers to analyze the role of ROS in ceramide generation. In contrast to untreated BAECs, which showed a significant increase in ceramide formation following exposure to radiation, no ceramide formation was seen in cells pretreated with ROS scavengers, implicating ROS generation in endothelial dysfunction and the salivary gland hypofunction.

The effects of PDE-5is on endothelial function have also been studied in other medical conditions. Balarini et al<sup>55</sup> studied the effects of sildenafil on endothelial function in an ApoE<sup>-/-</sup> mouse model of spontaneous hypercholesterolemia. They found that sildenafil completely restored the impaired acetylcholineeNOX-mediated vasodilator response in the aortic ring of ApoE<sup>-/-</sup> mice. Furthermore, the NOX-inhibitor apocynin significantly

increased endothelial relaxation and sensitivity to acetylcholine in untreated ApoE<sup>-/-</sup> mice.<sup>55</sup> In another study, Musicki et al<sup>56</sup> demonstrated that continuous sildenafil treatment in transgenic sickle cell mice promotes activation of endothelial NO synthase, an enzyme catalyzing NO production. And yet another study showed that sildenafil restored endothelial NO synthase activity and reduced ROS production in mice with second hand smoke-induced ED.<sup>57</sup>

Reduced NO bioavailability is considered one of the main characteristics of endothelial dysfunction, since NO is paramount both in the initiation and maintenance of erections.<sup>58</sup> Future in vivo studies using mice with orthotopic prostate tumors should establish whether sildenafil treatment increases NO bioavailability after radiotherapy without interfering with tumor control.

We believe the strengths of this study to be the rigorous conduct of the experiments using a well-established endothelial cell line and new state-of-the-art methods to measure O<sub>2</sub><sup>•-</sup> and ONOO<sup>-</sup> generation. This is the first study identifying radiation-induced ONOO<sup>-</sup> generation intracellularly, within the endothelial cells, and demonstrating a mechanism of action for sildenafil protection of radiation-induced microvascular dysfunction. From a limitation standpoint, as in all in vitro experiments, equating the sildenafil concentration to serum levels in the human model is a challenge; however, this preliminary analysis sets the stage for the conduct of future such studies to aid us in elucidating the potentially protective effects of PDE-5i on erectile function after prostate radiation.

In summary, the present study demonstrated that sildenafil protects endothelial cells from radiation-induced oxidative stress by reducing NOX-mediated ROS generation. In addition, we have shown that sildenafil was able to inhibit the proapoptotic ASMase/ceramide pathway, resulting in reduced endothelial apoptosis. These studies provide a potential mechanism to Zelefsky et al analyzed penile Doppler ultrasonography in prostate patients with postradiation ED, where it was demonstrated that the majority of patients presented with atherogenic ED.

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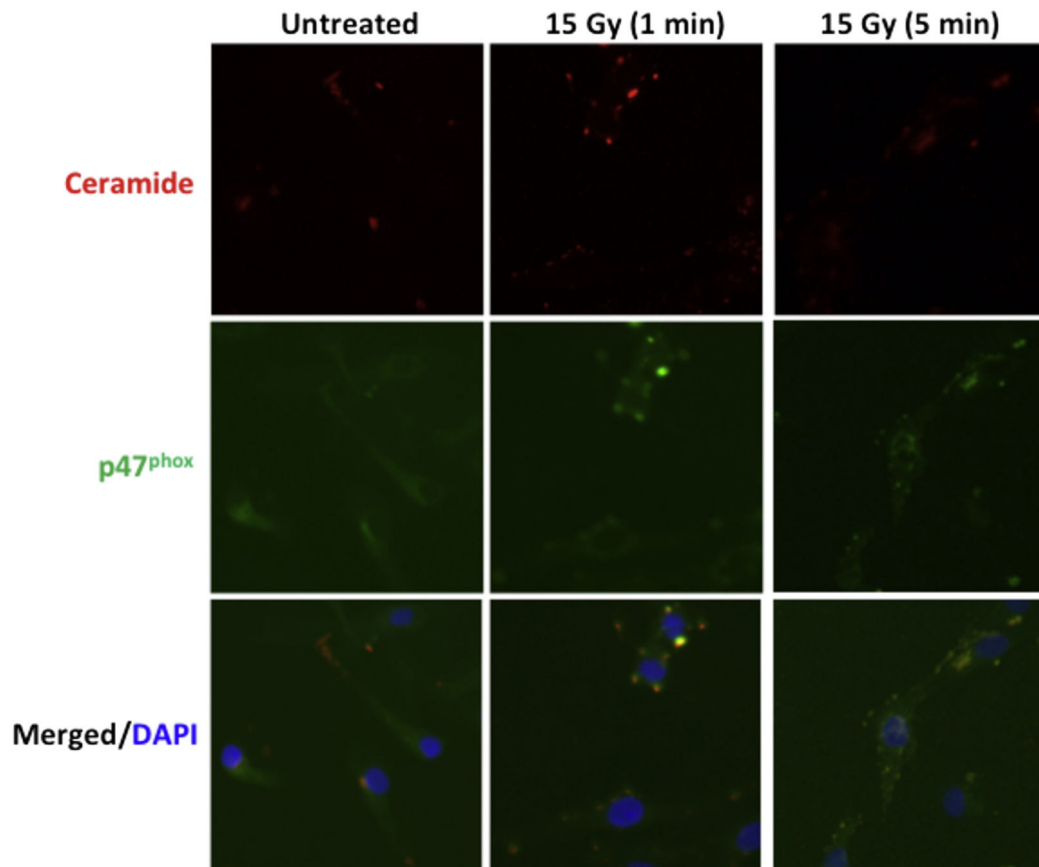
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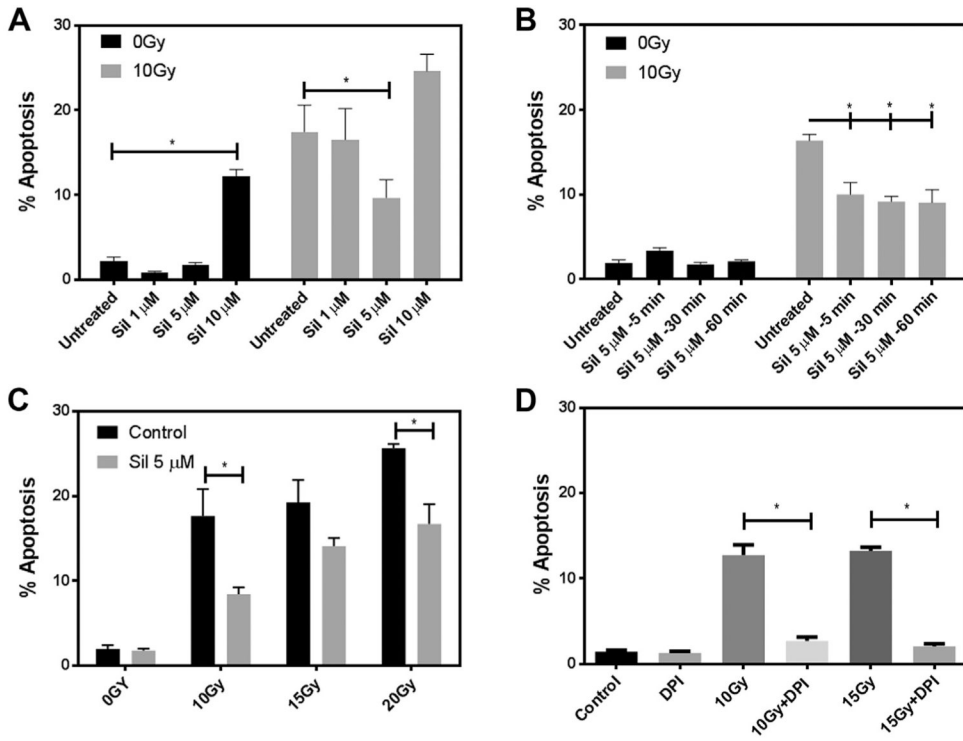
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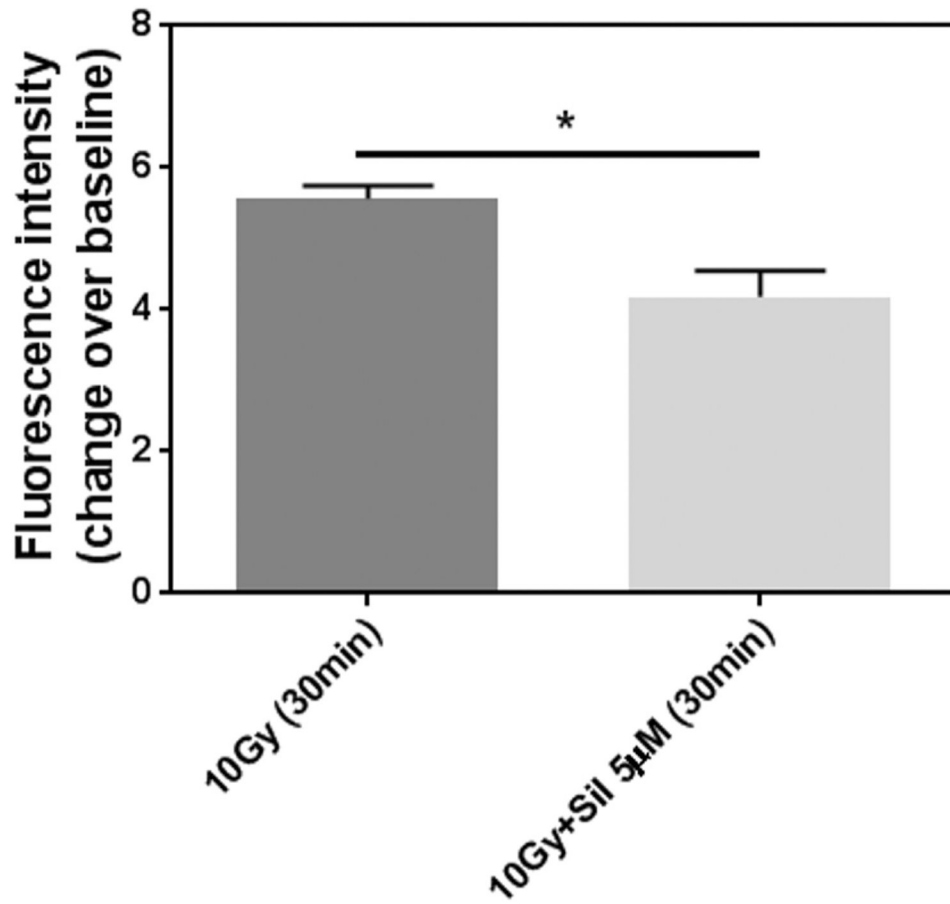
**Figure 1.**

NOX activation in response to radiotherapy in BAECs. Using confocal microscopy and staining endothelial cells with anticeramide antibody (red) and anti-p47<sup>phox</sup> (green), we showed radiation-induced co-localization of ceramide with p47<sup>phox</sup> as early as 1 minute postradiation, with a decrease after 5 minutes. The images were taken by microscope at 400× magnification. BAECs = bovine aortic endothelial cells; NOX = NADPH oxidase.

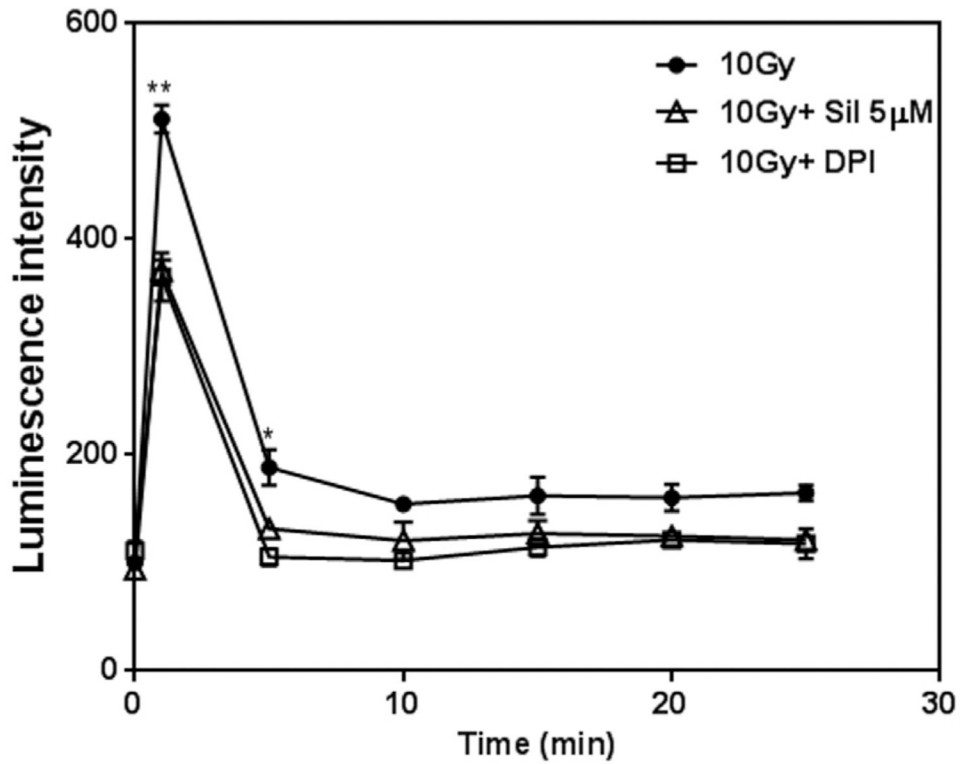




**Figure 2.** Sildenafil and NOX-inhibitor DPI (10  $\mu$ M) inhibit radiation-induced apoptosis in BAECs. Endothelial apoptosis was scored 8 hours after irradiation using the bis-benzimide trihydrochloride staining. Panel A shows sildenafil doses between 1 and 10  $\mu$ M added 5 minutes before irradiation to 10 Gy. Panel B shows sildenafil 5  $\mu$ M added between 5 and 60 minutes before irradiation to 10 Gy. Panel C shows sildenafil 5  $\mu$ M added 5 minutes before irradiation to 10–20 Gy. Panel D shows DPI (10  $\mu$ M) added 30 minutes before irradiation to 10–15 Gy. Data are expressed as mean  $\pm$  standard error from three independent experiments. \* $P$  < .05 of sildenafil-treated BAECs compared with untreated BAECs. BAECs = bovine aortic endothelial cells; DPI = diphenyleneiodonium; NOX = NADPH oxidase.

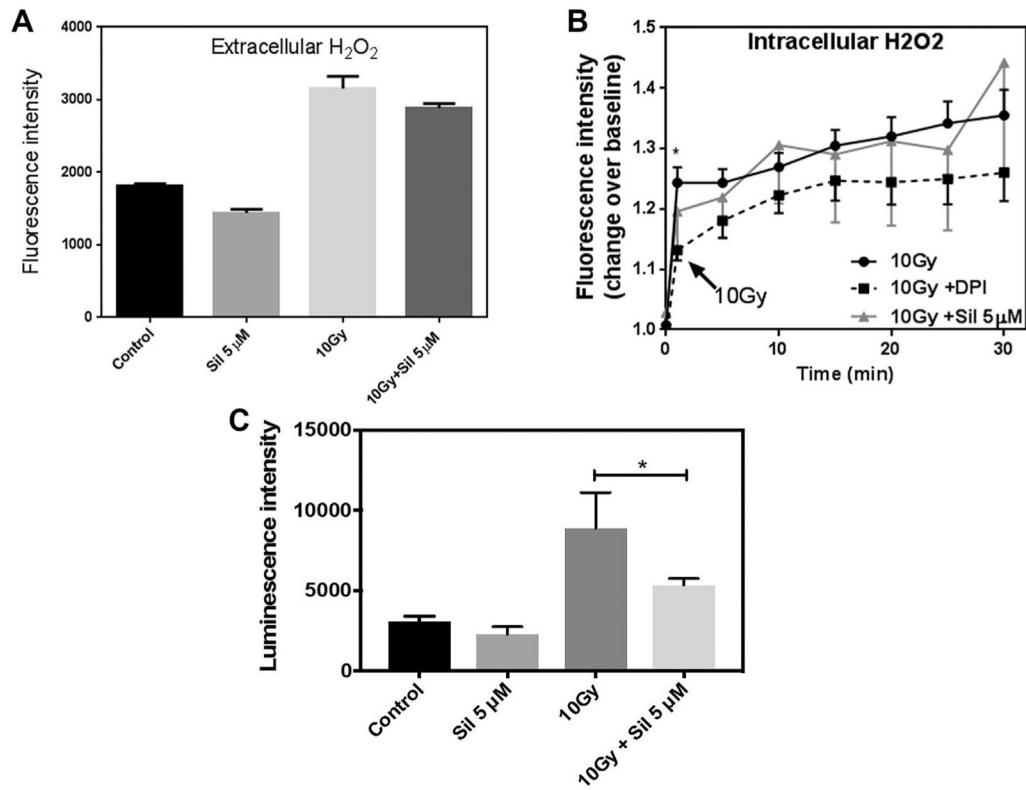


**Figure 3.** Sildenafil inhibits general ROS production in BAECs irradiated to 10 Gy. Sildenafil ( $5 \mu\text{M}$ ) was added 5 minutes before exposure to 10 Gy. Cellular ROS production assessed using the DCF-DA after irradiation with 10 Gy and 30 minutes incubation subsequently. Data are expressed as mean  $\pm$  standard error from 3 independent experiments.  $*P < .05$  of sildenafil-treated BAECs compared with untreated BAECs. BAECs = bovine aortic endothelial cells; DCF-DA = 2',7'-dichlorofluorescein diacetate assay; ROS = reactive oxygen species.



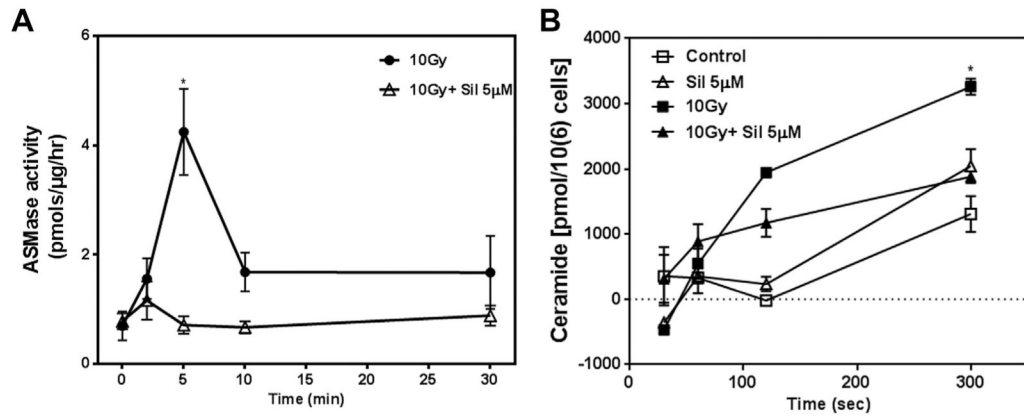
**Figure 4.**

Sildenafil and NOX-inhibitor DPI (10 mM) inhibit  $O_2^{\bullet-}$  production in BAECs after exposure to 10 Gy. Sildenafil and DPI (10  $\mu$ M) were added 5 and 30 minutes before exposure to 10 Gy, respectively. Data are expressed as mean  $\pm$  standard error from 3 independent experiments.  $**P < .01$  of sildenafil-treated and DPI-treated BAECs compared with untreated BAECs.  $*P < .05$  of DPI-treated BAECs compared with untreated BAECs. BAECs = bovine aortic endothelial cells; DPI = diphenyleneiodonium; NOX = NADPH oxidase.



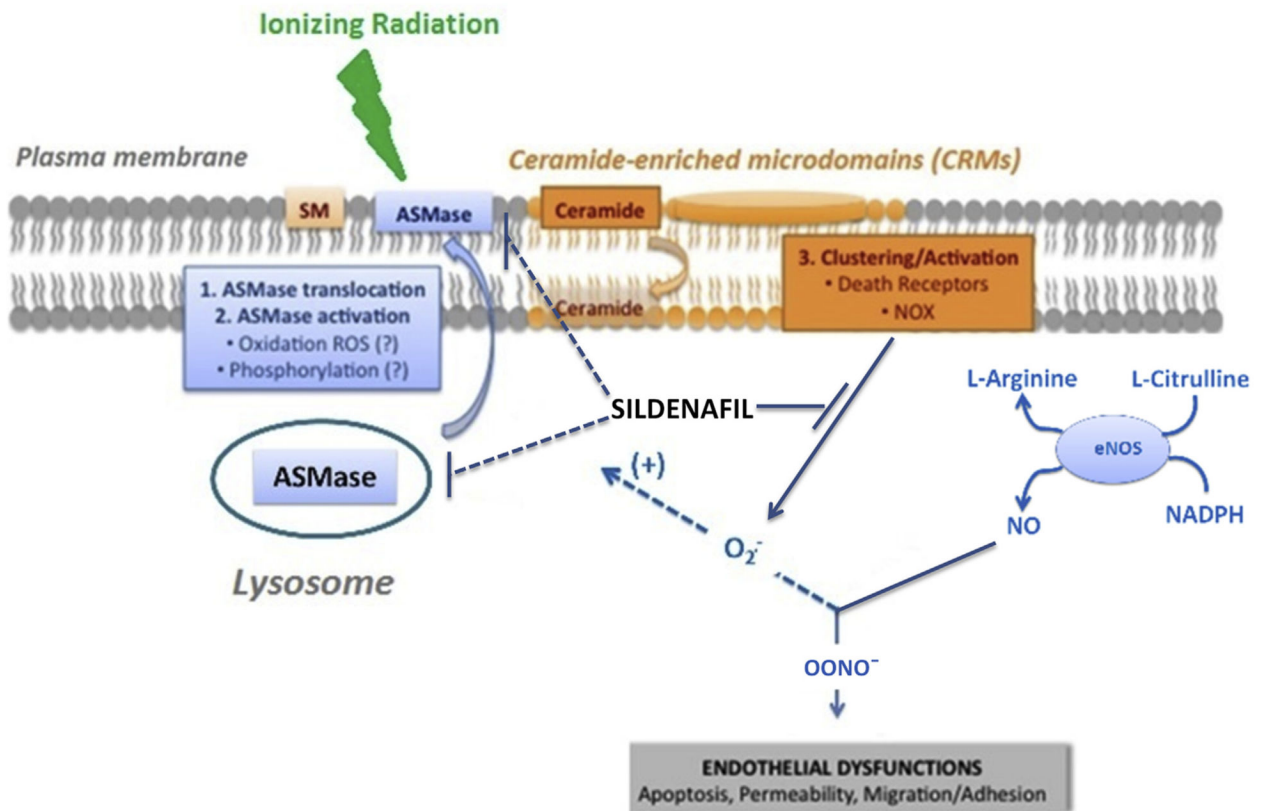
**Figure 5.**

Sildenafil at 5  $\mu$ M has no effect on extracellular or on intracellular H<sub>2</sub>O<sub>2</sub> in BAECs after exposure to 10 Gy. Sildenafil and DPI (10  $\mu$ M), were added 5 and 30 minutes before exposure to 10 Gy, respectively. DPI (10  $\mu$ M) inhibits intracellular H<sub>2</sub>O<sub>2</sub> fluorescence intensity at 2 minutes after exposure to 10 Gy. Panel A shows extracellular H<sub>2</sub>O<sub>2</sub> was measured using the Amplex Red assay (Molecular Probes) after 10 Gy irradiation and 30 minutes of incubation. Panel B shows intracellular H<sub>2</sub>O<sub>2</sub> was measured using HyPer transfection (Evrogen). Periodic images of transfected cells were taken at baseline and after 10 Gy irradiation. Data are expressed as mean  $\pm$  standard error from 3 independent experiments. \* $P$  < 0.05 DPI-treated BAECs compared with untreated BAECs. Panel C shows that sildenafil attenuates the production of intracellular peroxynitrite in BAECs after exposure to 10 Gy. Peroxynitrite levels were quantified by the chemiluminescent reporter PNCL, 10 minutes after 10 Gy of irradiation. Data are expressed as mean  $\pm$  standard error from 3 independent experiments. \* $P$  < .05. BAECs = bovine aortic endothelial cells; DPI = diphenyleneiodonium; H<sub>2</sub>O<sub>2</sub> = hydrogen peroxide.



**Figure 6.**

Sildenafil inhibits ASMase activity and ceramide generation in BAECs after exposure to 10 Gy. Panel A shows ASMase activity quantified by radioenzymatic assay using [<sup>14</sup>C-methylcholine] sphingomyelin. Panel B shows ceramide was measured using the diacylglycerol kinase assay after 10 Gy of irradiation. Data are expressed as mean ± standard error from 3 independent experiments. \**P* < .05 sildenafil-treated BAECs compared with untreated BAECs. ASMase = acid sphingomyelinase; BAECs = bovine aortic endothelial cells.



**Figure 7.**

Proposed sildenafil interaction within radiation-induced endothelial cell dysfunction via ASMase-ceramide-eNOS axis. This schematic model was adopted from work published by our collaborators (and with their and the journal permission) in response to cytokine activation in BAECs.<sup>25</sup> In response to radiation, ASMase containing lysosomes are driven to fuse with the cell membrane through soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins. ASMase is released and upon encountering its substrate, sphingomyelin, hydrolyses it to produce ceramide, within the outer leaflet of the plasma membrane. Ceramide, having fusogenic properties initiates the generation of CRMs. Subsequently, NOX subunits, such as gp91phox and p47phox, are aggregated, resulting in activated NOX via this process, and producing O<sub>2</sub><sup>•-</sup>. O<sub>2</sub><sup>•-</sup> may activate ASMase in a feed-forward mechanism, positive enhancing CRMs clustering and forming amplifications of this process. O<sub>2</sub><sup>•-</sup> coupling with NO generates another ROS, peroxynitrite (OONO<sup>-</sup>) but, most importantly depletes NO levels. Altogether, these processes constitute a redox signaling network or signalosome, resulting in endothelial dysfunction and impairment of endothelium-dependent vasodilation in coronary arteries. Sildenafil significantly inhibited ROS production in general: the immediate O<sub>2</sub><sup>•-</sup> production and the subsequent OONO<sup>-</sup> generation, in endothelial cells, by stopping the feed-forward activation of ASMase, ceramide generation, and NOX activation. ASMase =acid sphingomyelinase; BAECs = bovine aortic endothelial cells; CRMs = ceramide-rich microdomains; NOX = NADPH oxidase.