Different Effects of Photodynamic Therapy and γ-Irradiation on Vascular Smooth Muscle Cells and Matrix Implications for Inhibiting Restenosis

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Abstract—γ-Irradiation (γ-RT) and photodynamic therapy (PDT) are known to inhibit intimal hyperplasia. The common mechanism is that both modalities produce free radicals, but unlike γ-RT, PDT generates them through the absorption of light by photosensitizers. The purpose of this in vitro study was to assess the differences that PDT and γ-RT have on the fibroproliferative response after vascular injury by comparing their effects on vascular smooth muscle cells (SMCs) and on the extracellular matrix (ECM). Mitochondrial activity (tetrazolium salt), proliferation ([3H]thymidine incorporation), and the mechanisms of cell death (terminal deoxynucleotidyl transferase–mediated dUTP biotin nick end labeling [TUNEL] staining) were used to assess differences between PDT (100 J/cm²) and γ-RT (10 or 20 Gy) on SMC injury. The different effects on bioregulatory molecules were investigated by quantitating the proliferation of SMCs cultured with conditioned medium and on treated ECM. PDT of SMCs reduced proliferation and mitochondrial activity (0.5±0.75% and 1.7±4.25%, respectively, P<0.0001), whereas γ-RT of SMCs decreased cell proliferation but did not affect metabolic activity. Stimulation with calf serum of γ-RT–treated SMCs did not affect proliferation but increased mitochondrial enzyme activity (160±11%, P<0.0005). The conditioned medium, derived from PDT- but not γ-RT–treated SMCs, did not stimulate effector SMC proliferation compared with γ-RT–treated SMCs (16±4.1% versus 80±16.8%, P<0.0001). Apoptosis was the principle cytotoxic mechanism after PDT, whereas γ-RT cells were growth arrested but viable. PDT of the ECM reduced effector SMC proliferation compared with controls and γ-RT cells (18±6.5% versus 100±17.7% and 84±8.9%, respectively, P<0.0001). These data suggest that γ-RT and PDT may inhibit restenosis but by different mechanisms. The effects of PDT are more diverse and may result in improved outcome while avoiding the teratogenic exposure due to ionizing irradiation. (Arterioscler Thromb Vasc Biol. 1999;19:2154-2161.)

Key Words: restenosis ■ photodynamic therapy ■ ionizing irradiation

The development of surgical treatment of obstructive arterial disease represents an important achievement in the field of surgery. Within the past few decades, vascular surgery and novel percutaneous interventions have reached a high level of technical accomplishment; however, restenosis remains the major obstacle to satisfactory long-term patency after invasive vascular procedures.1 Restenosis is defined as luminal narrowing and may lead to the ultimate failure of the vascular reconstruction or intervention. The 2 major contributors responsible for this complex and multifactorial process are intimal hyperplasia (IH) and constrictive arterial remodeling.1,2

IH is the proliferation of smooth muscle cells (SMCs) and their migration from the media to the subendothelium, where deposition of significant quantities of extracellular matrix (ECM) also occurs.1 This process is thought to be mediated to a large extent by endogenous cell- and matrix-associated bioregulatory molecules.3 In addition to IH, studies have shown that injury-induced changes in the artery wall geometry, defined as arterial remodeling, may also play an important role in the development of restenosis.4 As in the arterial wound healing response, the total circumference of the artery may either decrease secondary to fibrotic contraction or increase secondary to compensatory dilatation. The multifactorial nature and the complexity of the events that culminate in restenosis indicate why it is still an unsolved problem.

Among numerous mechanical5,6 and pharmacological approaches,7,8 several current experimental strategies, such as gene therapy9 and ionizing irradiation, have been investigated to inhibit the occurrence of restenosis; however, to date, only stents and ionizing irradiation have been proven to clinically reduce this process.10–12 Ionizing irradiation generates free radicals by developing secondary electrons at the site of the absorbers, such as nucleic acids in the cell.13 Low-dose γ-irradiation (γ-RT) has been found to be effective in controlling nonmalignant fibroproliferative disorders such as heterotopic bone formation after hip replacement surgery.14 Because restenosis is a fibroproliferative response resulting

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from arterial injury, γ-RT is also being investigated to inhibit its development. γ-RT has been demonstrated to reduce experimental IH, and there are several clinical trials with promising results. However, there is limited understanding as to how γ-RT prevents restenosis.

Another promising treatment modality that may inhibit restenosis is photodynamic therapy (PDT). A technique that produces free-radical moieties by light activation of photosensitizer dyes. To perform PDT of the vascular wall, a photosensitizer is administered to the area of interest, which is then irradiated with visible light. The generation of localized free radicals, which exert their cytotoxic effects only at the site of light irradiation, results in changes in proteins and lipids. Inhibition of IH by PDT in balloon-injury models has been related to eradication of medial SMCs and alteration of its development.

The purpose of this study was to compare the 2 different modalities that exert their effect by free-radical generation, namely, PDT and γ-RT, on the response to vascular injury response. The effects on SMCs and endogenous cell- and matrix-associated bioregulatory molecules were studied to develop insight into how PDT and γ-RT modulate the vascular injury–induced fibroproliferative response.

Methods

Cell Culture

Primary cultures of bovine aortic SMCs and endothelial cells (ECs) were obtained from aortas of slaughtered calves by using the explant technique. Cellular identity was confirmed by indirect immunofluorescence and the use of an anti–α-actin antibody for SMCs and acetylated LDL for ECs (Biomedical Research Technologies, Inc.). Cells were kept in a 37°C, 5% CO₂ incubator and fed every 48 to 72 hours with complete Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (CS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.6 mmol/L L-glutamine (Gibco). On reaching confluence, the cells were passed at a 1:5 ratio by using 0.05% trypsin (Gibco), and subcultures from passages 2 through 6 were used for all experiments.

Preparation of the ECM

EC-derived ECM was prepared as previously described. In brief, the cells were seeded at a density of 1.5×10⁵ on 12-well plates, maintained at confluence for 6 to 8 days, and removed with 0.5% Triton X-100 (Sigma Chemical Co) and 20 mmol/L NH₄OH in PBS for 30 minutes. After being rinsed with PBS, the ECM with intact, associated growth factors was covered with PBS and stored at 4°C until used for experiments within 24 hours.

Preparation of Conditioned Medium

For preparation of conditioned medium, PDT-treated, γ-RT–treated, and untreated cells (positive control) were mechanically injured with a rubber policeman to release intracellularly located growth factors into the medium, as previously described, and incubated for 4 hours in 0.5% CS medium. The conditioned medium was collected and centrifuged at 2000 rpm for 5 minutes to remove cellular debris. The supernatant was assayed for SMC growth–promoting activity and compared with conditioned 0.5% CS medium from uninjured cells, which served as a negative control.

Photodynamic Therapy

PDT of the ECM was performed with the photosensitizer chloroaluminum sulfonated phthalocyanine (CASPC) diluted in PBS (5 μg/mL), and light was delivered by an argon-pumped dye laser using in vivo effective light dosimetry (Innova I and CR 599, Coherent; wavelength, 675 nm; thermoneutral irradiance, 100 mW/cm²; fluence, 100 J/cm² as described before). Controls included plates without the ECM and untreated ECM. In all experiments, ambient light exposure of the preparations was kept to a minimum.

To perform PDT of SMCs in culture, 2×10⁵ cells per well were seeded in complete medium on 12-well tissue-culture plates (Falcon, Becton Dickinson). After a 24-hour incubation, the medium was removed and rinsed, and the cells were incubated for 2 hours with CASPC (5 μg/mL) in PBS before light exposure. Immediately after PDT treatment, the CASPc was removed, and the cells were incubated in 0.5% CS medium for preparation of conditioned medium and incubated in complete medium (10% CS) or 100% CS for 24 hours in the proliferation and enzyme activity experiments.

γ-Irradiation

γ-RT of the ECM was performed using a 137Cs Mark I irradiator (J.L. Shepard & Associates) with an average dose of 2.54 Gy/min. Single, clinically relevant doses of 10 or 20 Gy were applied.

To perform γ-RT of SMCs in culture, 2×10⁵ cells/cm² were seeded in complete medium on 12-well tissue-culture plates. After a 24-hour incubation, the cells were irradiated with 10 or 20 Gy as described above. Immediately after γ-RT, the complete medium was removed, and the cells were incubated in 0.5% CS medium for preparation of conditioned media or in complete medium or 100% CS for 24 hours in the proliferation and enzyme activity experiments.

Mitochondrial Enzyme Activity

SMC mitochondrial enzyme activity was determined 24 hours after PDT treatment or γ-RT by using a viability colorimetric assay based on the bioreduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and an electron-coupling reagent (phenazine methosulfate) into a formazan (Promega). In brief, the solution was added to the cells and incubated at 37°C for 4 hours for color development, which was read at 490 nm. The optical density of untreated cells in 10% CS medium represented 100% enzyme activity, and the background color formation of MTS added to the 10% CS medium or 100% CS represented 0% mitochondrial enzyme activity. The optical density data from the treatment groups were fitted to a linear regression line obtained from the control groups to calculate percent mitochondrial enzyme activity. All data were corrected to absolute cell numbers.

Cellular Proliferation Assay

SMC proliferation 24 hours after PDT or γ-RT with complete medium and 100% CS was indirectly assessed using a mitogenic assay based on cellular [³H]thyidine incorporation. For the ECM experiments, SMCs were seeded at a density of 2.0×10⁵ cells in 0.5% CS medium on the prepared and treated matrices. Serum-poor medium (0.5% CS) was used to ensure that the cells or ECM was not subjected to serum constituents that might elicit cellular proliferation. After 24 hours of incubation at 37°C, 2.5 μCi of [³H]thyidine was added to the medium and incubated with the cells for another 5 hours. For the conditioned-medium experiments, the effector SMCs were seeded on empty plates and after 24 hours of incubation, the medium was removed and the prepared conditioned medium was added together with [³H]thyidine for 24 hours.

After incubation, the medium was removed and the cell layer washed 3 times with PBS. Unbound thymidine was removed by washing with PBS. Subsequently, 0.5N NaOH was added to dissolve the cells. The solution was supplemented with 2.0 mL of liquid scintillation cocktail (Beckman Instruments, Inc.), and radioactivity was determined with an automatic scintillation counter (Beckman LS 3801). The resulting data, expressed as counts per minute, were normalized to the untreated control groups and reported as percentage of thymidine incorporation. All data were corrected to cell numbers, which were determined before measurement by 6 random microscopic field counts per well under a phase-contrast microscope (Zeiss IM35).
**Cell Morphology**

SMCs were seeded at a density of $2.0 \times 10^5$ cells per well in complete medium for 24 hours. PDT and γ-RT (20 Gy) were applied as described. Cell morphology 2 hours after PDT and γ-RT was determined by viewing the cultured cells under a phase-contrast microscope (Zeiss IM35) at a magnification of $\times 32$. Untreated cells served as controls.

**Terminal Deoxynucleotidyl Transferase–Mediated dUTP Biotin Nick End Labeling (TUNEL) Stain**

SMCs were seeded on 8 chamber tissue-culture treated glass slides (Falcon) at a density of $10^5$ cells/cm$^2$ and incubated for 24 hours in complete medium at 37°C. PDT and γ-RT (20 Gy) were performed as described. Thirty minutes and 1, 2, 4, 12, and 24 hours after treatment, the medium was removed, the slides were rinsed with PBS, and the remaining cells were fixed in 4% buffered formalin for 10 minutes. The fixed cells were rinsed with PBS and stored at $-20^\circ$C for use within 5 days. To visualize apoptotic cells, in situ end labeling was performed using a standard fluorescein Apoptag apoptosis detection kit (Oncor). In brief, after incubation with an equilibration buffer for 5 minutes, 50 μL of terminal deoxynucleotidyl transferase was added to the slides for 2 hours. After terminating the addition of digoxigenin-conjugated dUTP by immersion in a stop-wash solution, the slides were incubated with fluorescein carrying anti-digoxigenin antibody. The slides were then counterstained with 10 μL of propidium iodide/antifade (Oncor) and kept in the dark at 4°C until the time of analysis within 48 hours.

An Axiophot fluorescence microscope (Zeiss) was used for viewing the specimens. The propidium iodide counterstain was visualized using a filter of 450 to 490 nm for excitation and of 515 to 565 nm for emission. The apoptotic cells were visualized using a band-pass filter of 564 nm for excitation and a long-pass filter of 590 nm for emission. Random counts of all attached and apoptotic cells per microscopic field (×40) were performed.

**Statistics**

All data are expressed as mean±SD. For comparison of means between multiple groups, a 1-way ANOVA and Tukey’s honestly significant difference post hoc test for multiple comparisons were applied (Statistica, Statsoft). A probability value of <0.05 was considered significant.

**Results**

**PDT and γ-RT Effects on SMC Proliferation**

To determine the differences between PDT and γ-RT on cellular proliferation after mitogenic stimulation similar to that in a site of vascular intervention in vivo, [3H]thymidine incorporation into SMCs was assessed (Figure 1). Cellular proliferation of untreated, control SMCs more than doubled after 24 hours of stimulation with 100% CS (237±6% compared with 100±4.26%, P<0.0001). There was no significant cellular proliferation detected 24 hours after PDT either with or without serum stimulation (1.5±0.97% and 0.5±0.75%, respectively) compared with empty plates (0±0.85%). The proliferative response of γ-RT cells decreased significantly after treatment with 10 or 20 Gy (24±3.01% or 32±2.72%, respectively, P<0.0001); there was no significant increase after incubation with 100% CS could not be assessed.

**PDT and γ-RT Effects on SMC Mitochondrial Enzyme Activity**

To investigate the metabolic status of SMCs after different treatment modalities, mitochondrial enzyme activity was assessed with and without cellular stimulation with 100% CS (Figure 2). Metabolic activity almost doubled in untreated cells after incubation with 100% CS for 24 hours, from 100±10.3% to 182±10.4% (P<0.0001). No significant enzyme activity was detected after PDT compared with background color formation in medium without cells. There was no observed difference in mitochondrial enzyme activity...
between untreated controls and γ-RT cells (100±10% versus 106±5%), and after stimulation with 100% CS for 24 hours, a significant increase (161±11%, P<0.0005) in enzyme activity was noticed in γ-RT–treated cells.

**Functional Significance of PDT and γ-RT Effects on Intracellular Growth Factors**

To assess the influence of PDT and γ-RT on injury-associated growth factors, a mitogenic assay of conditioned medium was used (Figure 3). As expected, the conditioned medium of mechanically injured SMCs significantly increased SMC mitogenesis compared with the conditioned medium of uninjured cells (100±14.23% versus 0±4.04%, P<0.0001). PDT of SMCs before injury significantly decreased the growth-promoting activity of their conditioned medium (to 16±4.14%, P<0.0001). γ-RT at 10 or 20 Gy did not significantly affect the growth-promoting activity of untreated SMCs after injury (89±22.74% and 80±16.68%, respectively).

**Cell Morphology**

The morphological differences of PDT-treated cells, γ-RT–treated cells, and untreated SMCs were examined 2 hours after treatment (Figure 4a through 4c). Both untreated and γ-RT SMCs showed a spindle cell shape, without vacuoles, and intact intercellular connections. In contrast, the PDT-treated cells were contracted, with a rounded appearance and diminished intercellular contacts.

**Apoptosis**

To investigate the mechanisms of cell death after PDT and γ-RT at a dose used for inhibiting IH in vivo, a TUNEL stain to assess apoptosis was performed (Figure 5). Apoptotic cell death was found to be the major cytotoxic mechanism after...
PDT, whereas after γ-RT and in control cells, no significant difference in the number of apoptotic cells was observed (2±2.9% versus 1.8±2.2%). After PDT, the percentage of apoptotic cells in proportion to all attached cells was 82.6±10.5% (P<0.0001) after 2 hours and did not change significantly in the time frame of the experiment. Ninety-five percent of the entire cell population was shown to be detached 24 hours after PDT, whereas the cell numbers after γ-RT and in untreated cells did not change significantly (Figure 6).

PDT and γ-RT Effects on EC-Derived ECM
To determine and compare the effects of the photochemical reaction induced by PDT and the application of γ-RT on ECM-associated growth factors, the proliferation of effector SMCs on treated matrixes was assessed. PDT of the ECM inhibited effector SMC proliferation significantly (18±6.5%, P<0.0001), whereas γ-RT of the ECM did not significantly affect the mitotic activity of SMCs compared with untreated controls (84±8.9% versus 100±17.7%) (Figure 7).

Discussion
This study compared the effects of PDT and γ-RT at in vivo effective doses on several of the factors known to be involved in the development of restenosis. Both PDT and γ-RT produce free radicals at their site of application: PDT mostly by the conversion of the triplet state of the photosensitizer or conversion to singlet oxygen free radicals and γ-RT by the release of nuclear photons during transformation to a more stable state of the molecule. Both PDT and γ-RT at specific doses are effective in controlling nonmalignant fibroproliferative disorders. PDT is effective in the treatment of psoriasis and arthritis, whereas γ-RT inhibits keloid formation. Therefore, it is not surprising that these modalities are being studied to inhibit the occurrence of restenosis as a fibroproliferative response to arterial injury.

However, most of the mechanisms by which γ-RT inhibits restenosis remain unclear. In this study, γ-RT at 10 and 20 Gy resulted in a significant decrease in cell proliferation. When stimulated with 100% CS to simulate the environment after vascular interventions, cell proliferation did not increase significantly. However, as was demonstrated in cultured fibroblasts, γ-RT induces growth arrest in the G1 phase, which can be reversible. Thus, cells are able to regain their ability to divide after undergoing DNA repair, albeit not at baseline levels. Doses greater than the cytostatic dose of 20 Gy would be required to completely eradicate the SMC population. However, these cytotoxic levels of ionizing radiation have an unacceptably high risk of late complications. Although it is not known how γ-RT inhibits IH, it has been proposed that radiation-induced growth arrest of SMCs and the fibrotic reactions in the vessel wall, which may induce a barrier for proliferating periadventitial cells and growth factors, are responsible for inhibiting restenosis. Another explanation might be its effects on cells undergoing mitosis, by causing cytotoxicity during cell division and limiting proliferation by reducing the number of regenerating clonal progenitors.

In contrast, PDT in the applied dose led to complete cellular eradication of the arterial wall in vivo and was also effective in
eliminating significant mitotic activity, independent of cellular stimulation with 100% CS. Although the PDT-induced cytotoxic effects led to complete cellular eradication, no aneurysms were seen in vivo. These results were found to be due to the lack of inflammation and the structurally intact matrix. It has also been shown that decellularized arteries after PDT are repopulated with cells, without leading to IH. However, it has also been demonstrated that appropriate PDT dosimetry is crucial to prevent IH.

The mitochondrial enzyme activity and therefore, the metabolic status of the cell reacted differently to PDT and γ-RT. Because PDT causes cellular eradication, there is no significant metabolic activity. Although there is decreased proliferation after γ-RT, the metabolic activity of the SMCs did not decrease significantly and responded to high-serum stimulation. These findings indicate the ability of γ-RT cells to respond to growth factors in serum, with an increase in mitochondrial enzyme activity. Growth-arrested SMCs after γ-RT were still metabolically active at both doses used and can be stimulated. Further investigations are necessary to assess the clinical relevance of these findings, but it is well known that cells that survive γ-RT undergo repair processes.

**Figure 6.** Fluorescence photomicrographs of SMCs (a) 2 hours after PDT, (b) 2 hours after γ-RT, and (c) left untreated. TUNEL staining, scale bar is 10 μm. The propidium iodide counterstain (red) was visualized using a filter of 450 to 490 nm for excitation and 515 to 565 nm for emission. The corresponding apoptotic cells (green) were visualized using a band-pass filter of 564 nm for excitation and a long-pass filter of 590 nm for emission.
and can be restored to full cell function. Irradiated cells, even after exposure to 20 to 60 Gy, can still synthesize growth factors, which may contribute to the proliferative response after injury and lead to the pathogenesis of late fibrosis.34,35

SMCs in culture are known to contain several growth-regulatory peptides, such as basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF), and it has been shown that mechanical injury results in growth factor release.36 Considering the presence of several cell-associated factors that could potentially modulate the injury response, this study did not attempt to pinpoint which specific factors were affected by PDT or γ-RT. Mechanically injured and γ-RT cells released functionally active growth factors, which resulted in an increase in SMC proliferation, whereas PDT treatment did not increase SMC proliferation, suggesting inactivation of these factors. PDT has been known to inactivate cell-associated bFGF,15 which would support these findings. The clinical significance of the conserved growth factors after γ-RT is still unknown. However, these paracrine factors may stimulate SMCs and may participate in irradiation-induced fibrosis, which could initiate constrictive remodeling.37

The mechanisms of cell death have gained interest for preventing restenosis, because apoptosis has been recognized to play a role in the development of IH. Attempts have been made to induce apoptosis in injured SMCs to reduce the amount of IH.38 However, the role of naturally occurring apoptosis remains unclear. It is not known whether it has a positive effect on limiting the proliferative response to vascular injury or whether it only modulates the cellularity of lesions that produce obstruction, particularly those with evidence of more extensive fibroproliferative activity.39 Because there is complete cell eradication after PDT, the mechanisms of cell death after treatment were investigated in this study. It was demonstrated that apoptosis is the major mode of cell death after vascular PDT, based on TUNEL staining and cell morphology. This may explain the absence of inflammatory cells and therefore, the potent inhibition of onset of IH in vivo.20 Inflammatory cells accumulate in balloon arteries, where injured SMCs release their cytokines and growth factors.40 However, cells undergoing apoptosis shrink, lose their normal intercellular contacts, and subsequently exhibit dense chromatin condensation, resulting in nuclear and cellular fragmentation into small apoptotic bodies. These bodies are phagocytosed and digested by adjacent cells. Because there is no release of cytosolic contents into the intercellular medium during apoptosis, inflammation is not triggered.41 However, after γ-RT, there was no significant decrease in SMC numbers, and therefore, few apoptotic cells were identified. These observations match previous findings in an in vivo study, wherein no significant differences in the percentage of TUNEL-positive cells in irradiated porcine coronary vessels were found up to 7 days after balloon angioplasty,42 compared with unjured arteries.

In addition to the cellular effects, it is known that the ECM and its biologically active components, including PDGF, transforming growth factor-β, and bFGF, play an important role in the fibroproliferative response to vascular injury.43 The ECM and its composition may lead to changes in cell growth, behavior, and differentiation, all of which can significantly contribute to the development of restenosis after vascular injury.44 PDT effects on the ECM have been shown to be important in modulating the healing response after vascular injury by favoring reendothelialization but inhibiting SMC growth and subsequent inhibition of IH,53 without resulting in physical alterations.17 This study confirmed the inhibition of effector SMC growth on PDT-treated matrixes. However, appropriate doses of γ-RT on the ECM did not affect the proliferation of SMCs compared with untreated control matrixes. These findings suggest that only PDT, and not γ-RT, alters the biologically active ECM components that may contribute to the prevention of the fibroproliferative response after vascular injury.

Cell- and ECM-associated growth factors and mitochondrial enzyme activity were not significantly altered at both doses of γ-RT. Especially at low doses, as applied in this study, little or no influence of different quantities of ionizing irradiation have been described for gene expression of matrix proteins.45 It is likely that a dose-dependent response may occur at higher doses. However, these are not clinically applicable for inhibiting vascular restenosis and were therefore not applied.

A number of important issues regarding the use of γ-RT to prevent restenosis remain unresolved. These include possible induction of tumors due to mutagenic effects after irradiation, defining which component of the arterial wall serves as the target tissue for radiation, the minimal effective dose, and the maximum tolerable dose. Further studies to obtain more insight into the underlying mechanisms of inhibiting restenosis are needed to define the safety, efficacy, and the ultimate usefulness of ionizing-radiation therapy in vascular interventions.

Despite the limitations associated with transferring in vitro data to the in vivo situation, the current data show that both PDT and γ-RT affect SMC proliferation. Although this may be the major component to the problem of restenosis, further effects were noted after PDT. These include inactivation of matrix components known to stimulate SMC proliferation. Furthermore, with almost-complete apoptotic cell eradication, PDT may suppress IH at sites of arterial injury, whereas
with γ-RT, the cells can still respond to cytokine stimulation. The simultaneous yet more diverse effects of PDT on the different components of vascular injury provide a strong promise for favorable clinical outcomes; however, further clinical evaluation is still necessary.

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