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PII: S0891-5849(10)01413-9
DOI: doi: 10.1016/j.freeradbiomed.2010.12.008
Reference: FRB 10459

To appear in: *Free Radical Biology and Medicine*

Received date: 26 August 2010
Revised date: 7 December 2010
Accepted date: 8 December 2010

Please cite this article as: Binata Joddar, Rashmeet K. Reen, Michael S. Firstenberg, Saradhadevi Varadharaj, Joe M. McCord, Jay L. Zweier, Keith J. Gooch, Protandim attenuates intimal hyperplasia in human saphenous veins cultured ex vivo via a catalase-dependent pathway, *Free Radical Biology and Medicine* (2010), doi: 10.1016/j.freeradbiomed.2010.12.008

Protandim attenuates intimal hyperplasia in human saphenous veins cultured ex vivo via a catalase-dependent pathway.

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Abstract: Human saphenous veins (HSV) are widely used for bypass grafts despite their relatively low long-term patency. To evaluate the role of reactive oxygen species (ROS) signaling in intima hyperplasia (IH), an early stage pathology of vein graft disease, and to explore the potential therapeutic effects of up-regulating endogenous antioxidant enzymes, we studied segments of HSV cultured ex vivo in an established ex vivo model of HSV IH. Results showed that HSV cultured ex vivo exhibit ~3-fold increase in proliferation, and ~3.6–fold increase in intimal area relative to freshly isolated HSV. Treatment of HSV during culture with Protandim, a nutritional supplement known to activate Nrf2 and increase the expression of antioxidant enzymes in several in vitro and in vivo models, blocks IH and reduces cellular proliferation to that of freshly isolated HSV. Protandim treatment increased the activity of SOD, HO-1, and catalase, 3-, 7- and 12-fold, respectively and decreased the levels of superoxide ($O_2^{\cdot-}$) and the lipid peroxidation product, 4-HNE. Blocking catalase activity by co-treating with 3-amino-1,2,4-triazole abrogated the protective effect of Protandim on IH and proliferation. In conclusion, these results suggest that ROS-sensitive signaling mediates the observed IH in cultured HSV and that upregulation of endogenous antioxidant enzymes can have a protective effect.

Introduction: Although arterial grafts are the preferred conduit for bypassing occluded coronary arteries, human saphenous vein (HSV) grafts are also used. The 10-year patency of the internal mammary artery (IMA) used in CABG is ~90% while the patency of HSV is only ~50%. Among patent HSV, about half suffer from significant stenosis leaving only 25% of total grafted SV performing optimally [1]. Early changes occur in vein grafts within 2 weeks of placement and include intimal hyperplasia (IH), involving migration and proliferation of smooth muscle cells from the media into the intima. This initial IH is believed to predispose the vein graft to atherosclerosis and thrombosis [1]. Thus inhibition of IH is an attractive target for improving vein graft performance. Despite numerous pharmaceutical attempts, only aspirin within 1 day of surgery, which can lead to bleeding complications, and blood-lipid lowering treatments have improved HSV graft patency [2-6]. Thus, additional methods to improve the patency of HSV grafts are needed.

Oxidative stress is associated with various forms of cardiovascular disease (CVD) [7] including hypertension and atherosclerosis [8]. Elevated levels of ROS are also present in veins grafted into the arterial circulation [2, 9-10] where they must function in oxygen concentrations approximately three-fold higher than those they have experienced in the venous circulation. Thus antioxidant therapies might be useful for the treatment of CVD including vein graft failure. Clinical studies with the antioxidant vitamins (A and E) did not demonstrate vascular protective effects [2, 11-14] but several potential limitations of these compounds include their tendency to also act as pro-oxidants [15] and failure to partition into a lipid-rich environment of a vascular lesion [15]. Probucol, an anti-hyperlipidemic drug with antioxidant activity, and its derivative,

succinobucol, have been shown to reduce atherosclerosis and restenosis in some [16-18], but not in all clinical trials [19]. In contrast to supplementation with exogenous antioxidants, the induction of endogenous antioxidant enzymes has several theoretical advantages [20], but this approach has not been widely tested in the context cardiovascular disease. Protandim, a mixture of five highly synergistic phytochemicals, has been previously shown to activate the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2) and to elevate the levels of the endogenous antioxidant enzymes superoxide dismutase (SOD), catalase, and heme oxygenase-1 (HO-1) in healthy humans [20] and / or in vitro models [21-22]. The basis of the synergy among the ingredients of Protandim has been previously described [22]. Nrf2 regulates the expression of more than a thousand genes involved in such areas as antioxidant protection, metabolism of xenobiotics, ubiquitin/proteasome systems, stress response proteins, kinases and phosphatases, lipid metabolism, cell cycle and cell growth, as well as genes involved in immunity, inflammation, fibrosis, and cancer chemoprevention.

The ex-vivo culture of SV is a widely used system to study IH [23-29]. The major benefits of such an ex vivo culture model are that it allows much better control and monitoring of chemical and mechanical environments than permitted in vivo while allowing the study of whole-vessel behavior not feasible in cell culture. The use of human tissues, especially tissues from atherosclerotic patients, is a significant benefit as there are numerous examples of interventions for vascular disease developed and tested in animal models failing to demonstrate a clinical benefit [7, 30-34]. HSV cultured for 14 days showed development of IH similar to that evident in HSV grafts placed as arterial substitutes in vivo [35], [1]. We recently reported that the exposure of porcine saphenous veins to arterial levels of pO_2 during ex vivo culture, as would also occur

when they are grafted into the arterial circulation as a bypass graft, stimulates the observed IH and cellular proliferation and is associated with markers of oxidative stress [35-36]. To evaluate the role of reactive oxygen species (ROS) signaling and the potential therapeutic effects of up-regulating endogenous antioxidant enzymes on neointima formation in SV, we studied segments of human SV remaining after coronary artery bypass grafting.

Materials and Methods:

Human Vessel harvest and preparation: The Institutional Review Board at The Ohio State University approved all use of human tissue in this study and all patients provided written informed consent for tissue donation. Segments of the great-saphenous vein which were in excess or unused at the end of CABG were obtained from consenting patients. Veins obtained from patients with documented varicosities of the long-saphenous vein and communicable diseases such as HIV and hepatitis B, C were excluded. All vessels were removed by an atraumatic no-touch endoscopic harvest protocol and washed in heparinized-saline after harvest. No veins discarded clinically for poor quality were used for this study. Vessels (HSV) were transported to the laboratory in a gas-impermeable chamber containing ~100 cc of culture medium (DMEM with low glucose (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 µg/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 25 mM HEPES buffer solution) pre-equilibrated with 95 mm Hg pO_2 and balance air and pre-warmed to 37ºC. Following this the vessels were immediately set up for culture as described later.

Ex-vivo organ culture: HSV were cultured inside sterile 50 mm petri dishes (Nalgene Nunc, Fisher Scientific). The surface of the cell culture dishes was scratched using sterile forceps to facilitate vessel adherence. Intact vessels selected for culture were cleaned of adherent adipose tissues. Cleaned vessel segments usually 6-8 cm length were cut open longitudinally and attached onto Petri dishes with the luminal surface exposed upwards and the adventitial side facing downwards. Any portions of HSV containing valves were excluded from culture to avoid false interpretation of valve material as IH in histomorphometric analysis. Vessels were cultured in 10 ml of low-glucose DMEM (Invitrogen, Carlsbad, CA) and housed at 37ºC in an oxygen-, carbon dioxide-, nitrogen- and humidity-controlled incubator (NUAIRE 4950) for 14 days. Medium was replaced every 2 days. HSV segments were cultured ex vivo at 95 mm Hg (~arterial pO_2 in vivo) with and without Protandim.

Protandim Supplementation: Protandim is a mixture derived from five botanical sources [*Bacopa monniera*, *Silybum marianum* (milk thistle), *Withania somnifera* (Ashwagandha), *Camellia sinensis* (green tea), and *Curcuma longa* (turmeric)] [20]. The alcohol extract of Protandim was prepared by shaking 675 mg of Protandim with 16.8 ml of 95% ethanol overnight at 4 °C and centrifuging at 5000 rpm (4 °C) for 5 min, and the extract (40 mg/ml) was stored at −80 °C. The addition of this ethanolic extract of complete Protandim to the cell culture medium resulted in a Protandim concentration of 10 μg/ml. Cultured HSV not receiving Protandim were treated with the same volume of 95% ethanol used in the Protandim treated group. N-acetyl cysteine (NAC, 20 mM, Sigma), a glutathione precursor, was dissolved in water for supplementation to select HSV cultures to compare its effects with that of Protandim supplementation. Both Protandim and NAC was added every 2 days, when medium was replaced

throughout the 14-day culture period. No separate vehicle controls were conducted for NAC which was dissolved in water, for which the maximum volume of supplementation per 10 ml of culture media never exceeded 50 µl.

Histomorphometric analysis: Upon removal from culture, the vessel sections were fixed in formalin overnight, dehydrated and embedded in paraffin. 5-8µm sections were cut and mounted on glass slides. H&E staining (Richard-Allan Scientific, MI) was done to analyze the vessel morphology and to detect changes caused due to culture of HSV ex vivo. Elastin staining (Accustain Elastic Stain, Sigma) was conducted according to manufacturer's instructions, and the intimal and medial areas of vein cross-sections, which were delineated by the external (EEL) and internal elastic lamina (IEL), were measured using Image J (NIH). Intimal area was determined by quantifying the area above the IEL in HSV cultured for 14 days. A minimum of 15 histological sections at 100 µm intervals were examined and the intimal area was determined. In each histological section, 4 separate areas were analyzed, quantified and averaged. Proliferating cells were identified with monoclonal mouse PC 10 antibody recognizing proliferating cell nuclear antigen/HRP (PCNA, DAKO). The in situ cell death detection, POD kit (TUNEL, Roche Applied Science, Indianapolis, IN) was used as directed. TUNEL- and PCNA-stained sections were counterstained with DAPI (Vector Laboratories, Burlingame, CA). The number of TUNEL- and PCNA -positive cells was expressed as a percentage of the total number of DAPI-stained cells counted on images of stained vein sections.

Detection and quantification of superoxide ($O_2^{-\cdot}$)*:* Production of superoxide ($O_2^{-\cdot}$) in HSV cultures was analyzed semi-quantitatively [37]. Briefly vein sections from 0, and 14-d cultures

were frozen in optimum cutting temperature compound media (Tissue-Tek; Sakura Finetechnical, Tokyo, [20]). Cryosections of 10 µm were prepared using Cryostat CM3000 (Leica Microsystems, Inc., Deerfield, IL), and treated with dihydroethidine (DHE; 1 µM) for 30 min at 37°C under dark conditions and imaged within 5 min. A group of sections were incubated with polyethylene glycol conjugated superoxide dismutase (PEG-SOD), which is used to scavenge superoxide ($O_2^{-\cdot}$) [38]. Amounts of superoxide ($O_2^{-\cdot}$) present were assessed using conversion of non-fluorescent DHE to fluorescent ethidium bromide. Images were obtained with a Nikon Eclipse TE 2000-S microscope (Nikon Corporation, Japan), with an excitation of 488 nm and emission of 574 to 595 nm. Fluorescent images were analyzed using Image J.

Analysis of 4-hydroxynonenal (4-HNE) by immunostaining and Western blotting: 4-HNE is highly reactive and forms stable 4-HNE adducts which can be detected and measured. 4-HNE immunostaining was done on paraffin-embedded sections to semi-quantitatively compare the extent of lipid peroxidation in HSV vessel sections using polyclonal antibodies recognizing 4-HNE adducts (Bethyl Labs, Montgomery, TX). Previously frozen tissue from fresh and cultured HSV was homogenized and lysed for Western Blot analysis using 4-HNE polyclonal antibodies (Axxora, San Diego, CA) to detect and quantify levels of 4-HNE produced in HSV sections.

Assay for enzymatic activities of catalase, SOD and HO-1: Previously frozen tissue from fresh and cultured HSV was homogenized and lysed to detect and quantify catalase, SOD and HO-1 activity. Catalase assay was based on the reaction of the enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide (H_2O_2) (Cayman Chemical, Ann Arbor, MI). SOD activity was assessed by measuring the dismutation of superoxide ($O_2^{-\cdot}$) radicals generated

by xanthine oxidase and hypoxanthine (Cayman Chemical, Ann Arbor, MI). This assay measures the combined activity of all three types of SOD (SOD1, SOD2 and SOD3). HO-1 activity assays utilized an ELISA method which had a mouse monoclonal antibody specific for HO-1 (Assay designs-Stressgen, Ann Arbor, MI), pre-coated on the wells of the immunoassay plate.

Inhibition of catalase activity: To inhibit the activity of catalase, we added 3-amino-1,2,4-triazole (AMT), (Sigma, MO, USA), an inhibitor specific for catalase at doses of 1, 5, 10, 20 and 50 μM solution in DMSO to HSV cultured ex vivo. The effect of varying the dose of AMT on cytotoxicity was analyzed by TUNEL assay and counting DAPI-stained cells.

Visualization and measurement of protein expression for catalase: CAT expression was visualized in histological sections by immunofluorescence and the corresponding protein levels were analyzed by Western blots. Both of these methods employed a catalase specific antibody (peroxisome marker for catalase, Abcam, Cambridge, MA, USA).

Statistical analysis: All data are reported as means ± SD. Data from paired study designs were analyzed using Student's paired t-test. For each experiment or condition n ≥6 HSV obtained from different patients were used. A value of $p<0.05$ was considered statistically significant.

Results:

Protandim inhibits the formation of IH and the increase in cellular proliferation in HSV cultured ex vivo: Elastin staining revealed that HSV cultured ex vivo exhibited IH (Fig. 1B, Fig 2A) and medial thickening (Fig. 2C) accompanied by increased cellular proliferation (Fig. 2B, D) compared to freshly-isolated (uncultured) HSV (Fig. 1A, 2A-D). These changes were attenuated by adding Protandim to HSV cultured ex vivo (Fig. 1C, Fig 2A-D). Supplementation with NAC also inhibited the increase in intimal and medial areas as well as cellular proliferation (Fig. 2). All freshly-isolated and cultured HSV showed normal cellular staining and an intact endothelium (Fig 1D, E, F and Fig. 6D, E, F) as shown by H&E staining. HSV cultured ex vivo in the presence of vehicle controls (50 µl of 95% EtOH) exhibited IH and levels of cellular proliferation similar to the no-Protandim controls (Fig. 2A,C).

Protandim attenuates rise in superoxide ($O_2^{-\cdot}$) levels in HSV cultured ex vivo: Freshly-isolated HSV exhibited relatively low levels of endogenous superoxide ($O_2^{-\cdot}$) as observed by DHE fluorescence (Fig. 3A). HSV cultured ex vivo showed increased levels of fluorescence (Fig. 3B), indicating elevated levels of ROS/superoxide ($O_2^{-\cdot}$) (~3.5 ± 0.5 fold, Fig. 3G). DHE fluorescence was blocked by addition of PEG-SOD, indicating its dependence on superoxide ($O_2^{-\cdot}$) (images

not shown). Addition of Protandim attenuated the increase in superoxide ($O_2^{-\cdot}$) in HSV cultured ex vivo (Fig. 3C) to levels comparable to freshly-isolated HSV (Fig. 3A). The pattern of DHE staining was consistent with nuclear staining by DAPI (Fig. 3D, E, F).

Protandim attenuates rise in lipid peroxidation (4-HNE) levels in HSV cultured ex vivo: To determine if IH in HSV cultured ex vivo is accompanied by lipid peroxidation, the levels of 4-HNE in HSV were assessed. The intensity and nuclear localization of 4-HNE protein adduct immunoreactivity in HSV cultured ex vivo was greater than freshly-isolated HSV (Fig. 4B versus A). Addition of Protandim reduced 4-HNE adducts immunoreactivity (Fig. 4C) to levels similar to that of freshly=isolated HSV (Fig. 4A). Western blot analysis showed that the addition of Protandim reduced 4-HNE adduct intensity ~87% as compared to 4-HNE adduct intensity detected in HSV cultured ex vivo (Fig. 4D).

Protandim enhances increases the activities of HO-1, total SOD and catalase in HSV cultured ex vivo: Addition of Protandim to HSV cultured ex vivo enhanced the activities of the endogenous antioxidants analyzed, HO-1, SOD and catalase ~7-fold (Fig 5A), ~3-fold (Fig 5B) and ~12-fold (Fig 5C), respectively relative to freshly-isolated HSV. The Protandim extract itself did not have detectable levels of HO-1, SOD or catalase activity. HSV cultured without Protandim had activities of HO-1, SOD and catalase similar to freshly-isolated HSV.

Addition of a catalase inhibitor attenuates the ability of Protandim to inhibit IH and cellular proliferation in HSV cultured ex vivo: We hypothesized that the Protandim-induced increase in catalase activity might be involved in Protandim's ability to inhibit IH. In order to block this

Protandim-induced increase in catalase activity, we added AMT, a specific catalase inhibitor [39]. Higher (50 µM and 20 µM) but not lower doses of AMT, increased the percentage of TUNEL-stained cells relative to freshly isolated HSV or HSV cultured without AMT (Table 1). Based upon these cytotoxic effects, both 50 µM and 20 µM AMT were excluded from further experiments. Protandim drug vehicle controls (DMSO), were not cytotoxic as they showed similar TUNEL indices (2.5 ± 0.4) compared to HSV cultured ex vivo. When added to HSV cultured with Protandim, AMT blocked the ability of Protandim to inhibit the increase in cell proliferation (Fig. 6) or the increase in intimal area (Fig. 7). All HSV cultured ex vivo with Protandim and AMT showed cellular staining and an intact endothelium (Fig. 6D, E, F).

Addition of a catalase inhibitor attenuates the increase in catalase activity due to addition of Protandim in a dose-dependent manner in HSV cultured ex vivo.

Since Protandim resulted in the largest fold increase in catalase activity, we suspected that this increase in catalase activity might be required for its observed effects on IH. When added to HSV cultured with Protandim, AMT resulted in a dose-dependent reduction of catalase activity with 10 µM AMT reducing catalase activity in HSV cultured with Protandim to the same levels seen in freshly isolated HSV or HSV cultured without Protandim (Fig. 8).

Protandim enhances the protein level and immunofluorescent intensity of catalase in HSV cultured ex vivo: Results from Western blots showed that catalase protein levels were significantly increased in HSV cultured ex vivo with Protandim compared to freshly-isolated HSV or HSV cultured ex vivo without Protandim (Fig. 9). Immunofluorescence revealed that catalase was expressed inthe intima, media and adventitia, thus likely the associated endothelial

cells, SMC, and fibroblasts (Fig 10A-C). Weak fluorescence was observed in freshly isolated and in HSV sections cultured ex vivo without Protandim (Fig. 10A, B). On the contrary, HSV cultured ex vivo with Protandim showed significant increase in the catalase staining intensity (Fig 10C, D).

Discussion:

The major findings of these studies are the following. 1) Treatment of HSV with Protandim or NAC blocks both IH and medial thickening as well as the increased cellular proliferation in an established ex vivo model of the early stages of vein-graft disease. 2) Protandim treatment results in a significant increase in activity of catalase, HO-1, and SOD, which is accompanied by a decrease in superoxide ($O_2^{\cdot-}$) levels and the lipid peroxidation product, 4-HNE. 3) Blocking catalase activity by co-treating with AMT abrogated the protective effect of Protandim on IH and proliferation. 4) Treatment with Protandim increased the protein levels of catalase in comparison to untreated veins.

The ability of Protandim to completely block IH and reduce cellular proliferation in HSV harvested from individuals undergoing coronary artery bypass grafting makes it an attractive candidate for future consideration as a pharmacological treatment of vein-graft failure. In addition, understanding how Protandim blocks IH in HSV might provide insights in to the molecular mediators of vein graft disease and other potential pharmacological treatments or

targets. To explore the molecular mechanisms of Protandim's action, we quantified the activities of catalase, HO-1, and SOD, three endogenous antioxidant enzymes previously shown to be upregulated by Protandim [20, 22]. Protandim increased catalase, HO-1, and SOD activity by 12-, 7-, and 2.6-fold, respectively. These levels of enzyme up-regulation in HSV were in line with the ~10-fold increases in HO-1 promotor activitity, mRNA, protein, and activity in a neuroblastoma and pancreatic β-cell lines [22], but much greater than the ~30- and 54 % increase in SOD and catalase activity reported for erythrocytes from healthy humans taking Protandim daily [20] or the ~30- and 58 % increase in SOD and catalase activity in skin epidermal tissue of mice fed a diet supplemented with Protandim [21]. It is not clear if the relatively large up regulation of catalase, HO-1, and SOD activity in HSV are due to HSV being more responsive to Protandim than human erythrocytes or mouse epidermis, a greater effect of the effective concentration of Protandim in ex vivo system, or due to other factors. In our ex-vivo studies, we matched the dose of Protandim per volume to that used in the human studies, the concentration of Protandim in the culture medium was 10 ug/ml. Given the in vivo metabolic pathways, clearance routes, and tissue distribution variables that are not present in the ex vivo system, resulted in a greater upregulation of enzymes in our study. Regardless of the reason(s) for the greater up regulation of the antioxidant enzymes in the cultured HSV, our data demonstrate that Protandim, at a concentration that does not reduce cell viability, blocks IH and cell proliferation while up regulating the activity of three endogenous antioxidant enzymes.

Protandim-induced up regulation of catalase, HO-1, and SOD activity in HSV are associated with a decrease in superoxide ($O_2^{-\cdot}$) levels and 4-HNE. The decrease in 4-HNE may reflect a reduced rate of lipid peroxidation due to more efficient scavenging of superoxide ($O_2^{-\cdot}$) and

hydrogen peroxide, but it also likely reflects increased metabolism of 4-HNE by aldo-keto reductase family 1 member B10 (AKR1B10), a critical protein in detoxifying dietary and lipid-derived unsaturated carbonyls [40-41]. Protandim up regulates AKR1B10 as strongly as HO-1 in human vascular endothelial cells [J.M. McCord, unpublished observation]. In addition to serving as markers of oxidative stress, both superoxide ($O_2^{-\cdot}$) and 4-HNE have been shown to stimulate proliferation of smooth muscle cells. 4-HNE stimulates SMC proliferation by MAPK-dependent pathways [42-43]. Superoxide ($O_2^{-\cdot}$) is converted to H_2O_2 by several SOD isoforms [44]. H_2O_2 can stimulate the proliferation of isolated human vascular smooth muscle cells [45] and the hypertrophy of arteries in vivo [46].

Given the established role of H_2O_2 in SMC and vascular remodeling and the fact that Protandim resulted in a greater fold increase in catalase activity than SOD or HO-1, we investigated the role of increased catalase activity in mediating the Protandim-induced inhibition of SMC proliferation by supplementing AMT to the ex-vivo cultured medium. AMT is an established inhibitor of catalase activity [39] that inhibits catalase irreversibly by reacting with catalase-H_2O_2 complex I [47]. In our study, AMT had a dose-dependent effect on catalase activity with an IC_{50} of 8 μM. Over the same range of concentrations, AMT dose-dependently abrogates the protective effects of Protandim on IH (EC_{50}=6.6 μM) and proliferation (EC_{50} = 6.8 μM) suggesting that Protandim-induced increases of catalase activity are required for its effects thus linking a change in enzymatic activity to a whole-vessel response. The Protandim-induced increase in catalase activity in HSV is accompanied with increases in HO-1 and SOD activity, and very likely other proteins regulated by Nrf2, so though necessary, the increased catalase activity is potentially not sufficient for the observed effects in HSV. The notion that increased

catalase activity is necessary but not sufficient is consistent with the suggestion by others that increasing SOD activity in the absence of elevated catalase activity would not be atheroprotective [48].

Veins grated into the arterial circulation are exposed to pressures approximately 5-fold greater than that of their native venous environment. The increased wall thickening often seen in grafted veins might be an adaptive response to the increased pressure or wall stresses [28-29, 49], though from a structural perspective, the 1,600 mmHg burst pressure of the native SV is more than adequate for the arterial pressure [50]. Our recent publication demonstrates that dramatic increases in the medial area and SMC proliferation occurs in saphenous veins exposed to arterial pO_2 in the absence of increased pressure [51]. This pO_2-induced medial hypertrophy is blocked by Protandim and NAC.

Taken together these data are consistent with the following model. Cultured HSV have elevated levels of superoxide ($O_2^{-\cdot}$), which can potentially contribute to SMC proliferation by 4-HNE-dependent and H_2O_2-dependent pathways. Treatment with Protandim increases endogenous SOD activity, which would account for the observed decrease in superoxide ($O_2^{-\cdot}$) and 4-HNE in Protandim-treated vessels. Up regulation of catalase activity is required for the protective effects of Protandim in this model suggesting that Protandim may act by altering the expression of multiple enzymes in concert.

Acknowledgments: This work was supported by the AHA (0655323B, 0555538U).

Author Disclosure Statement: JMM is a consultant to LifeVantage Corp. and has a financial interest in the company.

Sources of Funding: This work was supported by AHA 0555538U and 0655323B to K.J.G and HL63744, HL65608 and HL38324 to J.L.Z.

List of Abbreviations: 3-amino-1,2,4-triazole (AMT), human saphenous vein (HSV), 4-hydroxynonenal (4-HNE), heme oxygenase-1 (HO-1), Neointimal hyperplasia (IH), reactive oxygen species (ROS), superoxide dismutase (SOD), superoxide ($O_2^{-\cdot}$).

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Figure legends:

Fig 1. Protandim inhibits the rise in intimal area in HSV cultured ex vivo. Elastin and Van Gieson's stained histological sections of freshly isolated veins, and veins cultured with and without Protandim. The veins were imaged with the lumen facing downwards. The left panel of Elastin (modified Van Gieson's) stained images shows the development of IH below the IEL in B. Note the IEL denoted as a dark line in A, B and C. The right panel of Hematoxylin and Eosin stained images shows cellularity and also presence of the endothelium.

Fig 2. Protandim inhibits the development of IH and cellular proliferation in HSV cultured ex vivo. Intimal-, and medial areas (A, C), and mitotic indices (intima and media, B,D) of freshly isolated HSV and those cultured ex vivo with and without Protandim. $*$ was $p<0.05$ relative to other groups marked with a #. There were no intragroup differences amongst subgroups in either # or *.

Fig 3. Protandim attenuates the intensity of ROS formation in HSV cultured ex vivo. Effect of Protandim-supplementation on ROS formation in HSV cultured ex vivo shown in images A-F. ROS staining was achieved by incubating tissue sections with DHE and nuclei were visualized by adding DAPI. In all images, the vessel lumen is facing downwards. Quantification of ROS fluorescent intensity shown in G. PEG-SOD was added to inhibit the production of superoxide and depiected in G. $*$ was $p<0.05$ relative to other groups marked with a #. No intragroup differences amongst subgroups marked with #. Vehicle controls for Protandim (DMSO) showed no difference compared to HSV cultured ex vivo without Protandim (not shown).

Fig 4. Protandim attenuates the intensity of 4-HNE adducts formed in HSV cultured ex vivo. Effect of Protandim-supplementation on 4-HNE adduct formation in HSV cultured ex vivo shown in images A-D. Black-brown staining indicate the presence of 4-HNE adducts. Shown in E are the band intensities of 4-HNE adducts obtained from western blotting, normalized with actin. $*$ was $p<0.05$ relative to other groups marked with a #. No intragroup differences amongst subgroups marked with #. Vehicle controls for Protandim (DMSO) showed no difference compared to HSV cultured ex vivo without Protandim (not shown).

Fig 5. Protandim increases activities of HO-1, catalase, and SOD in HSV cultured ex vivo. Effect of Protandim-supplementation on HO-1 abundance, and endogenous activities of catalase

and SOD in freshly isolated HSV and HSV cultured ex vivo with and without Protandim. ∗ was $p<0.05$ relative to other groups marked with a #. No intragroup differences amongst subgroups marked with #. Vehicle controls for Protandim (DMSO) showed no difference compared to HSV cultured ex vivo without Protandim (not shown).

Fig 6. AMT attenuates inhibition of intimal area induced by adding Protandim to HSV cultured ex vivo, in a dose-dependent manner. Elastin and Van Gieson's stained histological sections of HSV cultured with Protandim and varying amounts of AMT. The veins were imaged with the lumen facing downwards. The left panel of Elastin (modified Van Gieson's) stained images shows the development of varying amounts of neointimal development above the IEL. The right panel of Hematoxylin and Eosin stained images shows cellularity and also presence of the endothelium.

Fig 7. AMT attenuates inhibition of IH induced by adding Protandim to HSV cultured ex vivo. Intimal area and mitotic index of HSV cultured with and without Protandim or variable doses of AMT. ∗ was $p<0.05$ relative to other groups marked with a #. No intragroup differences were observed amongst sub groups in either groups marked with an # or an *. Vehicle controls for Protandim (DMSO) and AMT (DMSO) showed no difference compared to HSV cultured ex vivo without Protandim or AMT (not shown).

Fig 8. AMT attenuates increase in catalase activity induced by adding Protandim to HSV cultured ex vivo. Quantification of catalase activity in freshly isolated and HSV cultured ex vivo with Protandim and variable doses of AMT. ∗ was $p<0.05$ relative to other groups marked with a #. Vehicle controls for Protandim (DMSO) and AMT (DMSO) showed no difference compared to HSV cultured ex vivo without Protandim or AMT (not shown).

Fig 9. Protandim increases protein amounts of catalase expressed in HSV cultured ex vivo. Western blot to estimate protein levels of catalase by addition of Protandim to HSV cultured ex vivo. ∗ was $p<0.05$ relative to other groups marked with a #. No intragroup differences amongst subgroups marked with an # were observed. Vehicle controls for Protandim (DMSO) and AMT (DMSO) showed no difference compared to HSV cultured ex vivo without Protandim or AMT (not shown).

Fig 10. Protandim enhances catalase intensity in HSV cultured ex vivo. Immunofluorescence staining for visualization of catalase in HSV cultured by addition of Protandim shown in images A-C. In all images, lumen is facing downwards. Shown in D is the semi-quantitative estimation of amounts of catalase (from immunofluorescence) in the HSV cultured by addition of Protandim. ∗ was $p<0.05$ relative to other groups marked with a #. No intragroup differences amongst groups marked with an # were observed. Vehicle controls for Protandim (DMSO) and AMT (DMSO) showed no difference compared to HSV cultured ex vivo without Protandim or AMT (images not shown).

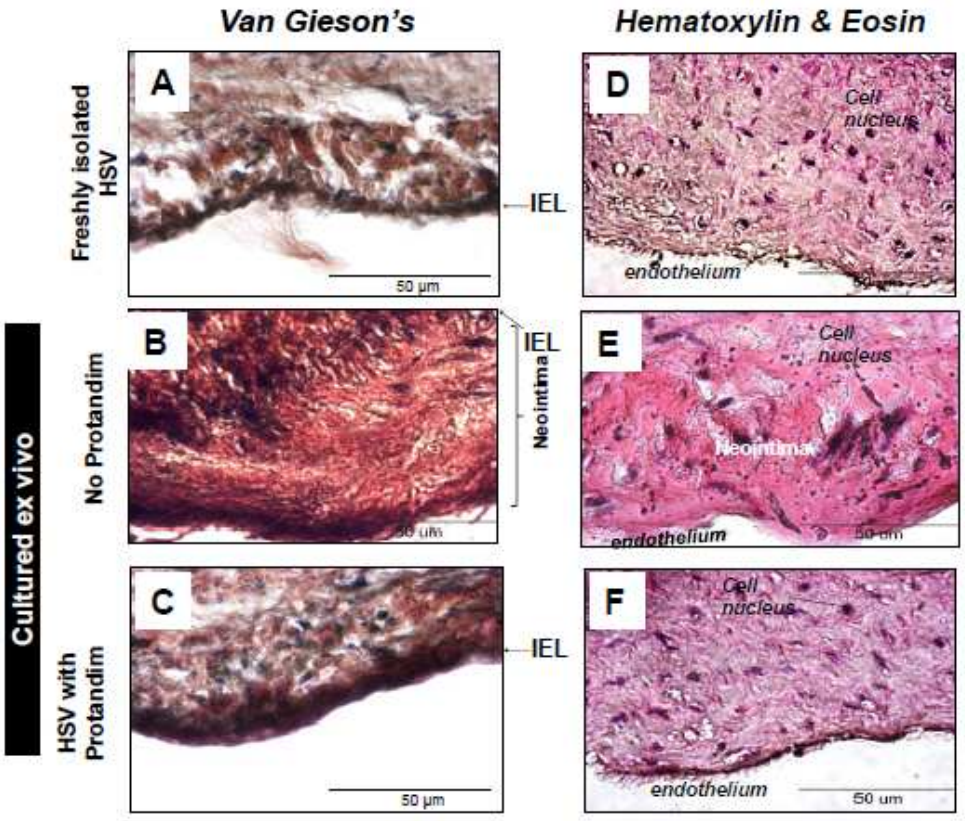
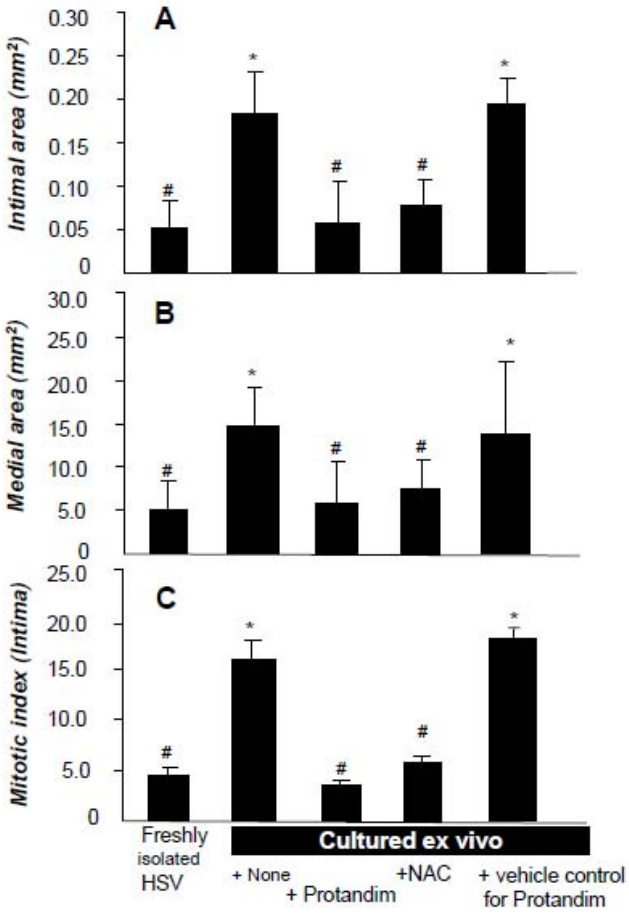


Figure 1.



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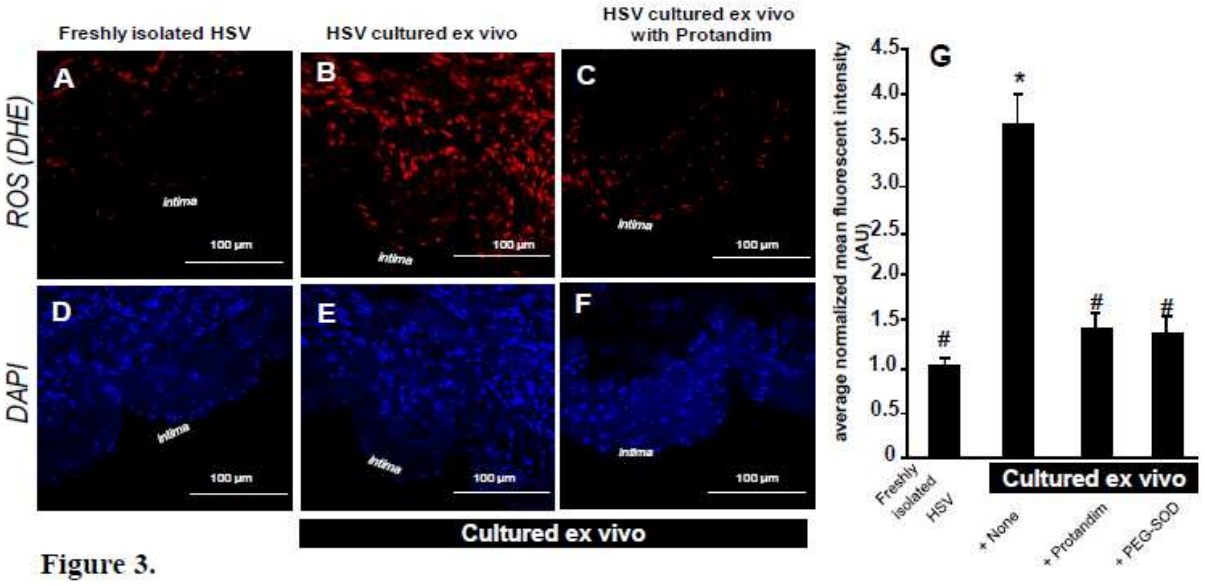

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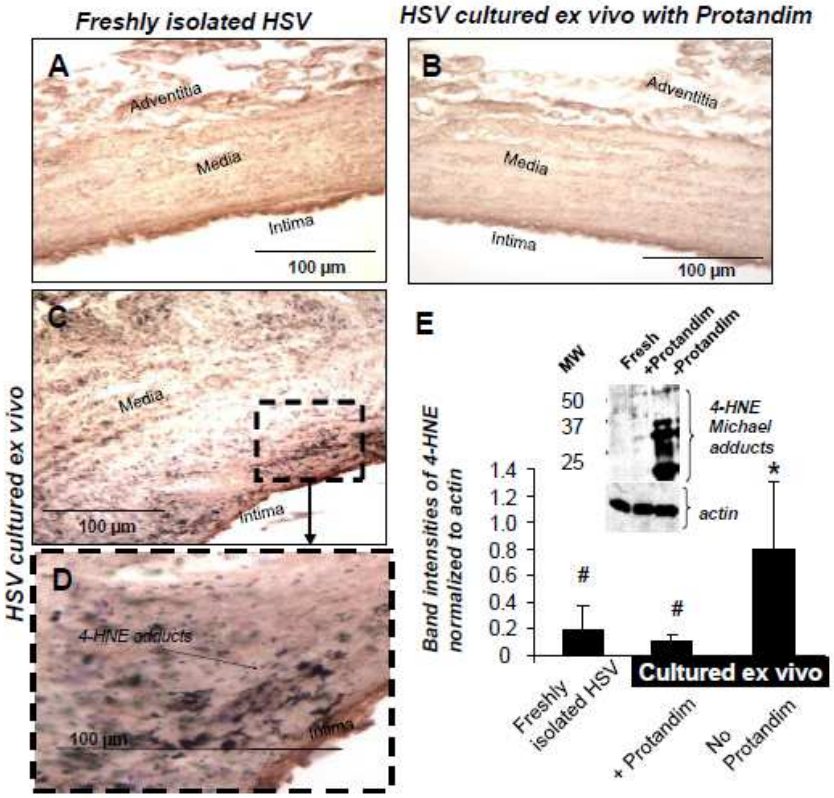

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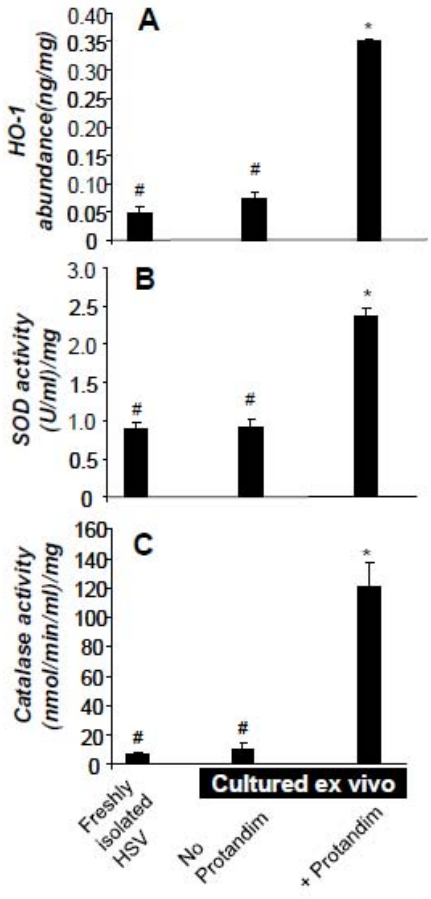

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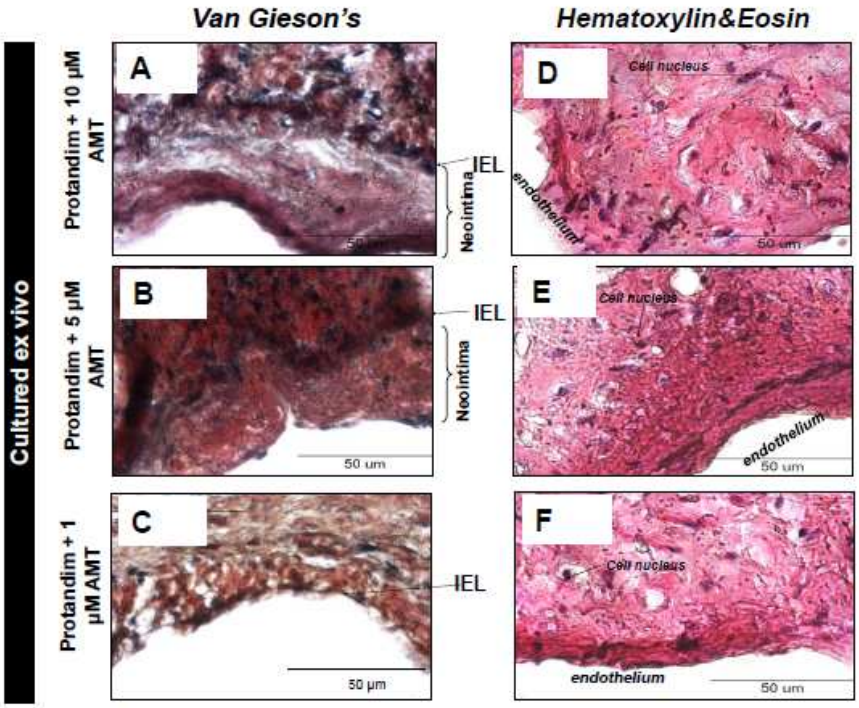

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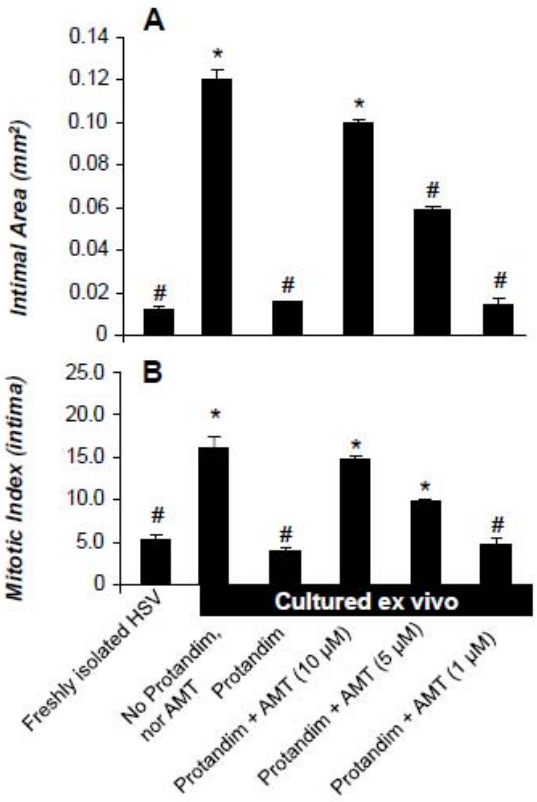

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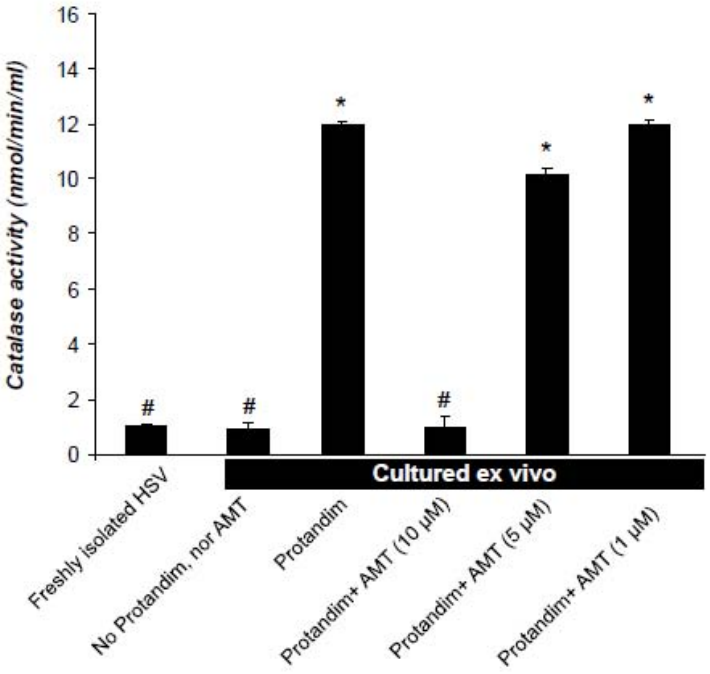

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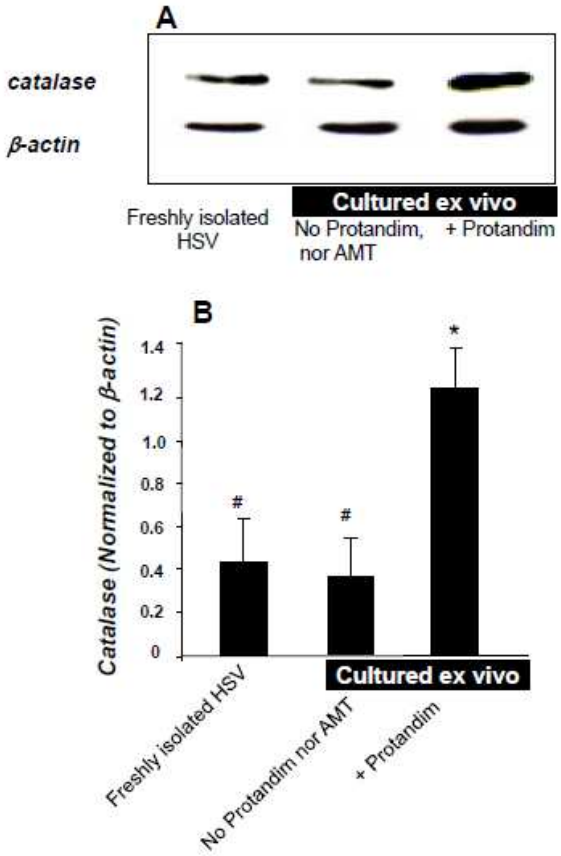

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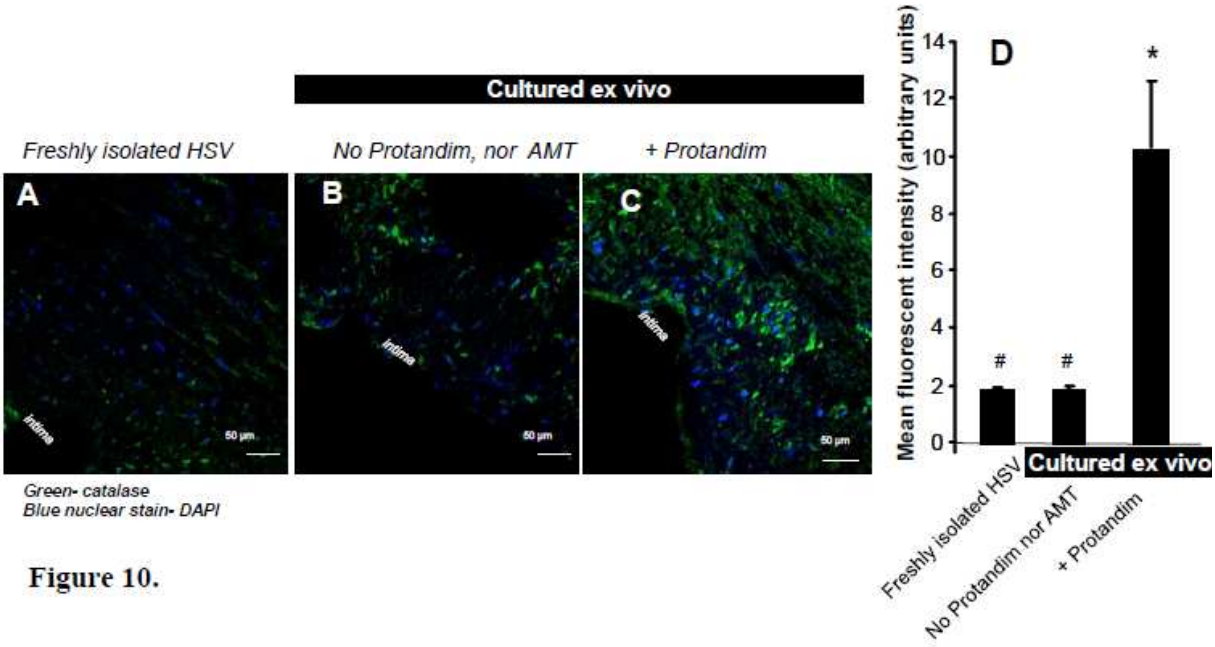

Figure 10.

Table 1. Apoptotic cell index (TUNEL) and DAPI (cell counts) for Protandim and AMT treated HSV sections cultured ex vivo.

	Apoptotic Cell Index (%) ($p<0.05$ relative to veins cultured ex vivo)	DAPI (#/mm^2)
Freshly isolated SV	0	Intima: 50 ± 10 Media: 60 ± 14
HSV cultured ex vivo	2.8 ± 0.3*	Intima: 95 ± 15* Media: 140 ± 12*
HSV cultured ex vivo + Protandim	2.4 ± 0.1*	Intima: 80 ± 10 Media: 90 ± 10#
HSV cultured ex vivo + Protandim + AMT (50 µM)	4.4 ± 0.1*#	Intima: 95 ± 17* Media: 140 ± 15*
HSV cultured ex vivo + Protandim + AMT (20 µM)	3.8 ± 0.1*#	Intima: 80 ± 17 Media: 120 ± 15*
HSV cultured ex vivo + Protandim + AMT (10 µM)	2.2 ± 0.2*	Intima: 90 ± 17* Media: 130 ± 15*
HSV cultured ex vivo + Protandim + AMT (5 µM)	2.2 ± 0.4*	Intima: 80 ± 10 Media: 90 ± 10#
HSV cultured ex vivo + Protandim + AMT (1 µM)	2.4 ± 0.1*	Intima: 40 ± 17# Media: 60 ± 15#

All data are shown as mean ± SD. $p<0.05$ was statistically significant. Groups marked with * were significantly different compared to freshly isolated HSV. Groups marked with # were significantly different compared to control HSV cultured ex vivo with FHSV excluded from this analysis.