Dietary antioxidants improve arteriogenic erectile dysfunction

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Introduction

Erectile dysfunction (ED) is defined as the persistent inability to achieve or maintain an erection sufficient for satisfactory sexual performance (Krane et al., 1989; Corona et al., 2004; Siroky & Azadzoi, 2004). Penile erection is a neurovascular phenomenon that requires dilation of penile vasculature, relaxation of smooth muscle, increased intracavernosal blood flow and normal veno-occlusive function (Saenz et al., 1988; Kim et al., 1991; Azadzoi et al., 1992). Arteriogenic ED results from haemodynamic impairment and chronic exposure of erectile tissue to ischaemia and hypoxia, nutrient deficiency and a lack of metabolic waste clearance (Grein & Schubert, 2002; Siroky & Azadzoi, 2003; Azadzoi et al., 2005). It is suggested that accumulation of endogenous nitric oxide synthase (NOS) inhibitors in the ischaemic penile corpus cavernosum may be involved in erectile tissue dysfunction.
and structural damage (Grein & Schubert, 2002; Siroky & Azadzoi, 2003; Azadzoi et al., 2005). Hypoxaemia and respiratory failure are increasingly recognized as causes of ED (Fanfulla et al., 2000). These conditions are known to involve oxidative stress caused by dysfunction of antioxidant enzymes and excessive production of free radicals such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radicals (Bello-Klein et al., 2001; Helmut, 2005). Oxygen-free radicals in the NO-rich tissues tend to combine to form peroxynitrite (O=NOO⁻), a highly cytotoxic product of O₂⁻ and NO radical reaction (Bello-Klein et al., 2001; Helmut, 2005; Qutub & Popel, 2008). Accumulation of these radicals results in lipid peroxidation, protein oxidation, DNA oxidation, decreased synthesis and bioavailability of endothelial and neuronal NO and upregulation of proinflammatory cytokines, growth factors and tissue-specific receptors (Bello-Klein et al., 2001; Helmut, 2005; Qutub & Popel, 2008).

An extensive, highly effective group of protective agents and defence mechanisms referred to collectively as the antioxidant defence system, acts to regulate oxidative reactions (Bello-Klein et al., 2001; Helmut, 2005). The antioxidant defence system includes both enzymes and antioxidants to prevent the start of oxidative damage and/or control its spread. There are also enzymes to repair oxidative damage, and mechanisms to target damaged molecules for destruction and replacement (Helmut, 2005; Qutub & Popel, 2008). Essential antioxidants are either endogenous (internally synthesized) or exogenous (ingested or administered; Helmut, 2005). They are typically categorized as scavenger antioxidants and preventive antioxidants. Antioxidants such as vitamins E and C have been used widely in clinical practice to protect the body from harmful free radicals (Kinlay et al., 2004). Other families of antioxidants with a more potent free radical scavenging capacity, such as polyphenols, were shown to prevent cardiovascular oxidative injury (Fuhrman et al., 1995; Hayek et al., 1997; Aviram et al., 2000; Aviram & Dornfeld, 2001). Consumption of dietary antioxidants with potent free radical scavenging capacities might also be effective in protecting the cardiovascular system (Fuhrman et al., 1995; Hayek et al., 1997; Aviram et al., 2000; Aviram & Dornfeld, 2001). Indeed, consumption of red wine, pomegranate juice, blueberry juice and green tea was shown to inhibit oxidative stress and prevent free radical injury (Fuhrman et al., 1995; Hayek et al., 1997; Aviram et al., 2000; Aviram & Dornfeld, 2001).

Our previous studies with a rabbit model of arteriogenic ED have shown that chronic penile ischaemia impairs erectile smooth muscle relaxation and leads to diffuse structural damage (Azadzoi et al., 1998, 2004). The goal of this study was to search for molecular and subcellular markers of oxidative stress in arteriogenic ED and examine the prophylactic role of dietary antioxidants.

**Materials and methods**

The selection of a dietary antioxidant

In a previous study, we examined the antioxidant potency of several known antioxidant beverages such as pomegranate juice, red wine, blueberry juice, cranberry juice, orange juice and green tea based on their free radical scavenging capacity using spectrophotometry (Azadzoi et al., 2005). Pomegranate juice demonstrated the highest antioxidant activity among the aforementioned beverages (Azadzoi et al., 2005). Therefore, in this study, we used a pomegranate extract liquid named POMXL supplied by POM Wonderful Inc. (Los Angeles, CA, USA). After expelling most of the juice from the pomegranate whole fruit, the remaining fruit including peels and membranes is collected and processed for seed removal before production of a puree. The puree is enzymatically treated and pomegranate polyphenols are concentrated via membrane system (Aviram et al., 2000; Aviram & Dornfeld, 2001). The resultant liquid is filtered to produce a pomegranate polyphenol extract. The obtained extract is then concentrated after passing through an evaporator and pasteurized. The final product contains 130 mg/mL of polyphenol antioxidants, as reported previously (Aviram et al., 2000; Aviram & Dornfeld, 2001). In our study, POMXL was mixed in drinking water. The desired concentrations were calculated based on body weight and daily water intake of the animals. We examined the effects of 1× concentration equivalent to 30 mg polyphenols per day, 2× equivalent to 60 mg polyphenols per day and 4× equivalent to 120 mg polyphenols per day in 3.5-kg rabbits. Drinking water without POMXL was used as the placebo.

**The arteriogenic ED model**

All animal studies were completed before December 2007 in accordance with the regulations outlined by our Institutional Animal Care and Use Committee. A total of 40 male New Zealand white rabbits were divided equally into control and treatment groups. In the treatment group, atherosclerosis-induced arteriogenic ED was developed by partial endothelial denudation of the iliac arteries under general anaesthesia, as described previously (Azadzoi et al., 1998, 2004). In brief, the animals were premedicated with intramuscular injection of 10 mg/kg ketamine and 0.05 mg/kg acepromazine, then anaesthetized with intramuscular injection of 35 mg/kg ketamine and 5 mg/kg xylazine. Anaesthesia was maintained with continuous administration of 1–2% isoflurane with oxygen.
Approximately half-inch incisions were made at the medial aspects of the right and left knees to expose the femoral arteries. A 3F Fogarty catheter (Edwards Lifesciences, Irvine, CA, USA) was passed through femoral arteriotomies to the bifurcation of the iliac arteries. Balloon of the catheter was inflated with 0.15 mL of saline and subsequently rotated and withdrawn to the respective femoral arteries. This manoeuvre was repeated thrice on each side. The animals received a 0.5% cholesterol diet for 4 weeks, then a regular diet until studied. The second group did not undergo arterial ballooning, received a regular diet and was used as the age-matched control. The balloon-treated and control groups of animals were assigned into four subgroups receiving 1× POMXL (five treated and five control animals), 2× POMXL (five treated and five controls), 4× POMXL (five treated and five controls) and placebo (five treated and five controls). After 8 weeks, the animals were anaesthetized as described earlier and the following studies were performed.

Measurement of penile blood flow and erectile activity

Penile intracavernosal blood flow was measured with a laser Doppler probe placed intracavernosally and connected to a laser Doppler flowmeter (Transonic Systems Inc., Ithaca, NY, USA; Azadzoi et al., 1998, 2004) Blood flow was recorded in the flaccid state of the penis and during nerve-stimulated penile erection. Erectile response to electrical stimulation of the cavernosal nerve was examined as described previously (Azadzoi et al., 1998, 2004). In brief, the cavernosal branch of the pelvic nerve was exposed at the lateral aspect of the prostate posterior to the rectum. Nerve stimulation was achieved via an electrode placed around the nerve. Systemic blood pressure, intracavernosal blood flow and intracavernosal pressure were recorded simultaneously using an eight-channel recorder (Astro-med Inc., Warwick, RI, USA). The quality of erection was assessed based on the ratio of mean intracavernosal pressure (MICP)/mean arterial pressure (MAP). After completion of the haemodynamic studies, the animals were killed with overdose of pentobarbital and the penises were removed. Erectile tissues were isolated and processed for the following studies.

Measurement of smooth muscle tension

Isometric erectile smooth muscle tension was examined in organ baths with physiological solution containing NaCl (118.3 mm), KCl (4.7 mm), MgSO4 (0.6 mm), KH2PO4 (1.2 mm), CaCl2 (2.5 mm), NaHCO3 (25 mm), Ca Na2 EDTA (0.026 mm) and glucose (11.1 mm). The solution was gassed with 21% oxygen, 5% CO2 and balance nitrogen. The pH of the solution was 7.4 and the temperature was maintained at 37 °C. Isometric tension was measured with a force transducer (Grass FT03; West Warwick, RI, USA). Tissues were stretched incrementally for approximately 2 h until optimal resting isometric tension for contraction was obtained. Endothelium-dependent relaxations to acetylcholine (Ach) were recorded in phenylephrine-contracted tissues.

Erectile tissue structure and image analysis

Masson’s trichrome staining of erectile tissue sections were performed according to the standard protocols. Histomorphometric image analysis of trichrome-stained tissues was performed in blind fashion using image analysis software (OPTIMAS 4.0 BioScan; BioScan, Edmonds, WA, USA), as described previously (Azadzoi et al., 2005). This technique is based on the calculation of red-stained smooth muscle area and blue-stained connective tissue area in randomly selected high power fields. Fifteen high power fields were selected and analysed for each animal. The percentage of smooth muscle was calculated for every high power field as the sum of the red-stained areas divided by the sum of all red- and blue-stained areas.

Enzyme immunoassay of oxidative products

Oxidative stress was assessed based on erectile tissue levels of the oxidatively modified product isoprostane-8-epi-Prostaglandin F2 alpha (PGF2α). Freshly dissected erectile tissues were equilibrated for 2 h in culture media (M199) with 10 μM fatty acid-free albumin at 37 °C. The media was exchanged with fresh media every half hour. After the last half hour of incubation, the media was collected and centrifuged at 4 °C at 1500 g for 15 min. The supernatant was utilized for measuring isoprostane-8-epi-PGF2α levels. All assays were in triplicate with commercially available enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI, USA). The quantity of isoprostane-8-epi-PGF2α was standardized as pg/mL of incubation media per mg wet weight of tissue per 30 min incubation.

Quantitative real-time PCR

Superoxide dismutase (SOD) and aldose reductase (AR) gene expression were examined in erectile tissues from the treated and age-matched control groups. Amplicon for SOD (Gene Bank: Z22644) and AR (Gene Bank: U12316) were prepared and processed for designing Taq-Man probes. Gene sequences were tested for repetitive sequence and masked where detected. Erectile tissue RNA was iso-
lated and 50 ng of RNA was processed for two steps quantitative real-time polymerase chain reaction (PCR; ABI Prism 7700; Applied Biosystems, Bedford, MA, USA). Taq-Man probes with forward and reverse primers with 18s probe as the internal control were used (Applied Biosystems). All samples were amplified in duplicate. Gene expression in the treated group was calculated as relative quantification to age-matched control values in folds.

Transmission electron microscopy

Erectile tissues were processed for immersion fixation in 2.5% glutaraldehyde and 2.5% paraformaldehyde, post-fixation in 2% osmium peroxide, dehydrated in graded ethanol and propylene oxide then embedded in Epon 812 (Shell Chemicals, Houston, TX, USA). Sections were stained with 1% toluidine blue, mounted on a 200-mesh copper grid and processed for stain with uranyl acetate and lead citrate for counterstaining. Tissue ultrastructure was examined using a Tecnai G² Spirit BioTWIN microscope (FEI Company, Hillsboro, OR, USA).

Statistical analysis

Data are expressed as mean ± standard error of the mean. All measured parameters in treated animals receiving POMXL or placebo were compared with control animals receiving POMXL or placebo, respectively. In addition, all measured parameters in the treated and control animals receiving POMXL were compared with their respective placebo groups. Significant differences were determined with analysis of variance (ANOVA) followed by post hoc comparisons. Statistical significance was determined at $p \leq 0.05$ level.

Results

Changes in penile blood flow

Arterial pressure of animals receiving 4× POMXL was significantly lower in comparison with animals receiving placebo, as shown in Fig. 1 ($p = 0.001$). Arterial ballooning produced diffused atherosclerotic occlusive disease and caused a significant decrease in intracavernosal blood flow in comparison with the age-matched control group (Fig. 1; $p = 0.008$). POMXL consumption significantly increased intracavernosal blood flow in both atherosclerotic and age-matched control groups in comparison with their counterpart placebo groups. However, intracavernosal blood flow in atherosclerotic animals receiving POMXL was significantly lower in comparison with control animals receiving either POMXL or placebo ($p = 0.002$). These observations suggest that POMXL intake significantly improved intracavernosal blood flow of atherosclerotic animals in comparison with atherosclerotic animals receiving placebo, but did not normalize to the blood flow levels recorded in control animals. There were no significant differences in intracavernosal blood flow

Figure 1 (A) Mean arterial pressure showing significant hypotension in animals receiving high concentration of dietary antioxidants. Asterisk indicates significant difference in 4× POMXL group vs. control group. (B, C) Effects of dietary antioxidants on intracavernosal blood flow during nerve-stimulated penile erection. Intracavernosal blood flow significantly increased in the erectile dysfunction (ED) group receiving POMXL in comparison with the ED group receiving placebo, but did not reach the levels recorded in age-matched control animals. Asterisks indicate significant differences in POMXL vs. placebo in the same group of animals.
among animals receiving 1×, 2× and 4× POMXL in the treated and control groups.

Erectile dysfunction

Electrical stimulation of the cavernosal nerve caused a marked increase in intracavernosal pressure leading to full erection in the control group (Fig. 2). ED characterized by a significant decrease in the percentage of MICP/MAP was evident in atherosclerotic animals (ED group). The percentage of MICP/MAP in the ED group receiving 1×, 2× and 4× POMXL significantly increased in comparison with the ED group receiving placebo ($p = 0.017$), but was lower than the levels recorded in control animals receiving POMXL or placebo (Fig. 2). POMXL had no significant effect on the percentage of MICP/MAP in control animals (Fig. 2). The data suggest that POMXL significantly improved erectile function in the ED group in comparison with the ED group receiving placebo, but did not normalize it to the levels recorded in age-matched control animals. There was no significant difference in erectile response among animals receiving 1×, 2× and 4× POMXL in the treated and control groups.

Changes in smooth muscle relaxation

Endothelium-dependent relaxation of erectile tissues from ED group receiving placebo was significantly less than the control group receiving placebo ($p = 0.023$; Fig. 3).

Figure 2  Effects of dietary antioxidants on penile erectile response to cavernosal nerve stimulation. Erectile response significantly increased in the erectile dysfunction (ED) group receiving POMXL in comparison with the ED group receiving placebo, but did not reach the levels recorded in control animals. Asterisks indicate significant differences in POMXL groups vs. placebo in the ED group.

Figure 3  Effects of dietary antioxidants on endothelium-dependent relaxation to acetylcholine. POMXL significantly increased endothelium-dependent relaxation in both the erectile dysfunction (ED) and control groups in comparison with their counterpart placebo groups, but did not normalize the relaxation differences between the ED and age-matched control groups. Asterisks indicate significant differences in POMXL vs. placebo in the same group of animals.
POMXL significantly increased endothelium-dependent relaxation in both ED and control groups compared with their placebos \((p = 0.027)\), but did not normalize the relaxation differences that existed between the ED group and age-matched controls (Fig. 3). There was no significant difference in erectile tissue relaxation among the 1×, 2× and 4× POMXL groups (Fig. 3).

**Structural damage**

Marked loss of smooth muscle and diffused fibrosis were evident in trichrome-stained erectile tissues from the ED group (Fig. 4). Histomorphometric analysis of Masson’s trichrome-stained penile sections from the ED group receiving placebo showed a significant decrease in the percentage of smooth muscle in comparison with the control group receiving placebo \((28.5 \pm 1.7 \text{ vs. } 44.6 \pm 2.1; p = 0.029)\). POMXL had no significant effect on smooth muscle content in the control group. The percentage of smooth muscle in the ED groups receiving 1×, 2× and 4× POMXL of 35.2 ± 1.5, 37.4 ± 1.9 and 34.9 ± 1.7, respectively, was significantly greater than the ED group receiving placebo \((p = 0.025)\), but remained significantly less than the control group receiving placebo \((p = 0.028)\). The data suggest improvement, but not normalization of structural damage in the ED group receiving POMXL.

**Oxidative products**

The levels of the oxidatively modified product isoprostane-8-epi-PGF2α was significantly greater in erectile tissue from the ED group receiving placebo in comparison with the controls receiving placebo, suggesting oxidative stress \((p = 0.001; \text{Fig. 5})\). Consumption of 1× POMXL was sufficient to diminish isoprostane-8-epi-PGF2α levels of the ED group to the levels measured in age-matched control group receiving placebo (Fig. 5).

**Molecular reactions**

Molecular changes typical of oxidative stress were evident in erectile tissues from the ED group. Significant upregulation of oxidative stress-responsive genes encoding SOD \((p = 0.005)\) and AR \((p = 0.02)\) were found in tissues from the ED group receiving placebo in comparison with the control placebo group, suggesting a defensive reaction against oxidative radicals (Fig. 5). POMXL at 1× concentration reversed these molecular changes of the ED group to the age-matched control levels (Fig. 5).

**Tissue ultrastructure**

Electron microscopy showed normal cellular and subcellular structures in erectile tissues from the control group (Fig. 6). However, marked thickening of endothelium, sporadic loss of endothelial layer surrounding lacunar spaces, swollen mitochondria with membrane deformation, loss of mitochondrial granules and increased caveolae on cell membrane were evident in erectile tissues from the ED group receiving placebo (Fig. 6). POMXL at 1× concentration appeared to protect mitochondrial and endothelial structural integrity and diminish caveolae levels in erectile tissues of the ED group (Fig. 6).

**Discussion**

Our data suggest oxidative stress in the ischaemic penis and protective role of dietary antioxidants against free radical incursion in arteriogenic ED. Our observations also imply that oxidative burden of ischaemic erectile tissue may exceed its antioxidant capacity and lead to oxidative injury. This may contribute to impairment of NO-mediated endothelium-dependent smooth muscle relaxation, and structural damage in the ischaemic penis (Grein & Schubert, 2002; Siroky & Azadzoi, 2003; Azadzoi et al.,

![Figure 4](image-url)  
**Figure 4** Samples of Masson’s trichrome-stained erectile tissues that underwent image analysis. Marked fibrosis evidenced by decreased smooth muscle content (stained red) and increased connective tissue (stained blue) was found in samples from the erectile dysfunction (ED) group receiving placebo. POMXL diminished fibrotic changes in the ED group in comparison with the ED group receiving placebo, but did not normalize it to the age-matched control levels.
We found that arterial occlusive disease of major penile arteries decreased erectile tissue blood flow, diminished intracavernosal perfusion pressure and impaired metabolic waste clearance from the erectile tissue. These changes were associated with cellular and subcellular reactions evidenced by accumulation of oxidatively modified products, upregulation of oxidation-sensitive genes encoding SOD and AR and ultrastructural alterations. Long-term POMXL consumption caused significant increases in intracavernosal blood flow, smooth muscle relaxation and erectile activity in the ED group, but did not normalize them to the levels recorded in age-matched control animals. Alternatively, POMXL therapy appeared more effective in inhibiting oxidative products, preventing molecular reactions and reversing ultrastructural changes of ischaemic erectile tissues to the control levels. These observations imply prompt and more efficient action of antioxidants on the molecular and ultrastructural alterations than on distinct pathological changes such as fibrosis and functional deficit. Full recovery from structural damage and dysfunction may require longer antioxidant therapy than the 8-week duration tested in our protocol. Partial restoration of erectile tissue structure and function may also suggest non-recoverable oxidative injury or the involvement of mechanisms beyond oxidative stress in arteriogenic ED. Hypotension, a side effect observed with 4× concentration of POMXL may relate to relaxation and dilation of the peripheral arteries. The slightly lower intracavernosal blood flow and MICP/MAP in animals treated with 4× vs. animals treated with 2× concentration may relate to hypotension in the 4× POMXL group.

In penile ischaemia, nutrient deficiency, inadequate antioxidant enzymatic activity, hypoxia and lack of

![Figure 5](image)

(A) Erectile tissue levels of the oxidatively modified product isoprostane-8-epi-PGF1α showing a significant increase in the erectile dysfunction (ED) group receiving placebo. POMXL decreased isoprostane-8-epi-PGF1α production of the ED group to the levels measured in age-matched controls. Asterisk indicates significant difference in ED vs. control group. (B) Upregulation of superoxide dismutase and androgen receptor gene expression in erectile tissues of ED group receiving placebo. POMXL diminished these molecular reactions to the age-matched control levels. Asterisks indicate significant differences in the ED vs. control group.

![Figure 6](image)

Electron microscopy of tissue samples from the control group receiving placebo, erectile dysfunction (ED) group receiving placebo and ED group receiving POMXL. The upper panel shows mitochondrial structural damage and increased caveolae on smooth muscle cell membrane of the ED sample receiving placebo. The lower panel shows thickened endothelium layer with increased caveolae and diffused subendothelial fibrosis in the ED sample receiving placebo. A marked decrease in these ultrastructural reactions is evident in tissue samples from the ED group receiving POMXL. White and black arrows point to mitochondria and endothelium lining of the cavernosal tissue, respectively. White double arrow shows subendothelial fibrosis. C points to the caveolae.
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perfusion to clear waste products constitute an environment conductive to formation and accumulation of oxidative radicals. Increased levels of erectile tissue isoprostane-8-epi-PGF2α and upregulation of oxidative stress-responsive genes in our ED model evidenced oxidative stress and intrinsic reaction to free radicals. In the healthy condition, penile free radicals are tightly regulated by physiological homoeostatic mechanisms and when formed are rapidly neutralized by biological antioxidants. In penile ischaemia, antioxidant defence system fails as a result of lack of nutrients and metabolic wastes accumulate. Under these conditions, free radical formation exceeds the antioxidant capacity of the penis allowing a vicious oxidative cycle to develop leading to protein oxidation, lipid peroxidation, DNA damage, leucocyte activation, cytokine production and disruption of the cellular and subcellular structures (Voss & Siems, 2006).

Cellular and molecular markers of oxidative stress in our ED model are consistent with changes reported in the ischaemic heart and vascular occlusive disorders. Interestingly, antioxidant therapy that is known to protect the cardiovascular system appeared to preserve erectile tissue ultrastructures and improve penile erection in our ED model. Clinical studies have shown that nearly 75% of men with coronary artery disease also suffer from ED (Solomon et al., 2003; Kloner et al., 2003). These studies have suggested that ED may be an early signal of impending coronary artery disease (Solomon et al., 2003; Kloner et al., 2003). Although the nature of this association remains unclear, the aetiology of both vascular disorders and ED involves oxidative stress risk factors such as smoking, diabetes, hypercholesterolaemia, atherosclerosis and hypertension (Solomon et al., 2003; Kloner et al., 2003). Endothelial cells are the primary target of these risk factors in both cardiovascular and penile erectile tissues. In the penis, endothelial cell injury and subsequent atherosclerosis and narrowing of the hypogastric-pudendal arterial bed result in diminished perfusion pressure, decreased arterial inflow to the lacunar spaces of the penile corpora cavernosa and lack of metabolic waste clearance (Grein & Schubert, 2002; Siroky & Azadzoi, 2003). The clinical consequences of these changes are progressive prolongation of the time to achieve full erection, lowered rigidity of the erect penis and ultimately erectile failure. This initiates free radical accumulation and incursion of endothelial cells leading to smooth muscle dysfunction and erectile tissue fibrosis.

Impairment of endothelium-dependent smooth muscle relaxation in arteriogenic ED may relate to lack of NO synthesis, decreased NO bioavailability or both. Oxygen free radicals tend to interact with NO to produce nitrosative radicals. This results in lack of NO bioavailability and upregulation of proinflammatory cytokines, fibrotic growth factors and tissue-specific receptors leading to impairment of endothelial cells and smooth muscle dysfunction (Ortiz et al., 2001; Aragno et al., 2003). In addition, free radicals can initiate a chain reaction of redox-sensitive signalling events involving kinases and transcription factors that regulate the expression of genes and proteins influencing smooth muscle relaxation. It has been shown that impairment of smooth muscle relaxation in atherosclerotic arteries results from destruction of NO by excessive endothelial superoxide production, rather than decreased synthesis (Bello-Klein et al., 2001). In our model, dietary antioxidant intake improved penile vasodilation evidenced by increased intracavernosal blood flow and increased erectile smooth muscle relaxation seen in the organ bath. The mechanism may involve neutralization of superoxides and other radicals in erectile tissue allowing NO to endure long enough to exert its vasodilating effects on penile vasculature and smooth muscle cells. Partial recovery of blood flow and smooth muscle relaxation may suggest non-recoverable pathological changes such as severe occlusive disease, physical loss of erectile tissue endothelial cells and smooth muscle atrophy in the chronically ischaemic penis.

Improvement of endothelium-dependent relaxation with dietary antioxidants such as POMXL may introduce newer therapeutic targets in ED. Endothelial cell dysfunction is one of the leading mechanisms of impaired erectile smooth muscle relaxation in ED (Fanfulla et al., 2000; Helmut, 2005). This is caused by the ubiquitous location of the endothelial cells in penile arteries and cavernous sinuses and the important role of endothelial cells in regulating neurotransmitter release and erectile smooth muscle tone. Endothelial cells, whether in the cavernosal artery or in the cavernosal sinuses, produce a variety of local paracrine factors to regulate smooth muscle tone. These factors include NO, angiotensin-converting enzyme, angiotensin, kinins, prostaglandins, endothelin and histamine (Kim et al., 1991; Azadzoi et al., 1992). Some of these substances, such as angiotensin, endothelin and certain prostaglandins are powerful vasoconstrictors, whereas others such as NO, certain prostaglandins and histamine are vasodilators. The endothelial cell is also the site of action of many factors and pharmaceutical agents (Kim et al., 1991; Azadzoi et al., 1992). Our data imply vulnerability of endothelial cells to ischaemic and oxidative radicals and the need for newer strategies to protect cavernosal endothelium in the ischaemic penis.

Thickened endothelial layer and diffused subendothelial fibrosis found in electron microscopy may contribute to impairment of endothelium-dependent smooth muscle relaxation in our ED model. Endothelial thickening and
increased subendothelial collagen is likely to alter diffusion and enhance degradation of NO during transit from endothelium to the underlying smooth muscle cells. Improvement of endothelium-dependent relaxation with dietary antioxidants in our model may relate to increased endothelium-derived NO synthesis, protection of NO from $\text{O}_2^-$ incursion, improvement of NO diffusion and a decrease in the production of endothelium-dependent constrictors such as endothelin-1 (ET-1; Ortiz et al., 2001). Numerous conditions characterized by impaired NO bioavailability have been associated with enhanced ET-1 synthesis, and vice versa, suggesting that these two factors may have a reciprocal regulation under the ischaemic conditions (Ortiz et al., 2001). An experimental model of hypertension has provided evidence that antioxidants diminish ET-1 production, while causing a parallel increase in NO production by the endothelial cells (Ortiz et al., 2001). Antioxidants may also act via angiotensin II, a key mediator of vasoconstriction. In hypertension, the conversion of angiotensin I to angiotensin II via angiotensin-converting enzyme (A-C-E) was shown to be regulated by free radicals and blocked by antioxidants (Ortiz et al., 2001). Pomegranate polyphenols have been shown to inhibit A-C-E in humans significantly (Aviram & Dornfeld, 2001).

The anti-fibrosis effect of POMXL in our ED model is consistent with the reported efficacy of antioxidants in attenuating tubulointerstitial disease in the rat model of nephrosis (Aragno et al., 2003). Oxidative stress and the formation of oxidative products in arteriogenic ED are presumed to play important roles in erectile tissue fibrosis. oxidative products are known to stimulate transforming growth factor (TGF)-$\beta$1 production and upregulate fibronectin gene expression (Ryu et al., 2004). It is thought that the newly produced TGF-$\beta$1 in response to oxidative stress stimulates fibronectin expression (Ryu et al., 2004). It has been shown that collagen degradation is affected by free radicals (Aragno et al., 2003; Ryu et al., 2004). In atherosclerosis, metalloproteinases secreted from macrophage foam cells degrade the cellular matrix (Aragno et al., 2003). It was shown that the antioxidant N-acetyl-cysteine diminishes metalloproteinase release and blocks its action (Aragno et al., 2003). Our observations with the ED model suggest a partial role of dietary antioxidants in preventing or delaying erectile tissue fibrosis in chronic penile ischaemia.

Upregulation of oxidative stress-responsive genes encoding SOD and AR implies an intrinsic defensive reaction of erectile tissue against free radicals in penile ischaemia. SOD is an enzyme that catalyses the dismutation of $\text{O}_2^-$ into oxygen and hydrogen peroxide and is known to repair cells and reduce the damage caused by $\text{O}_2^-$ (Peixoto et al., 2009). AR reduces cytotoxic aldehydes and glutathione conjugates of aldehydes derived from lipid peroxidation (Rittner et al., 1999). Inhibition of AR has been shown to increase oxidative injury and abolish the late phase of ischaemic preconditioning (Rittner et al., 1999). SOD and AR upregulation in our model may be an endogenous antioxidant reaction that seems to fail to prevent oxidative injury under the ischaemic condition. These molecular reactions in penile ischaemia may be limited to the transcription level and may not lead to protein synthesis in the ischaemic and hypoxic penis. Interestingly, POMXL prevented upregulation of SOD and AR gene expression in the ischaemic penis, possibly by eliminating oxidative elements involved in such molecular reactions. These observations suggest ineffective intrinsic antioxidant defence system in penile ischaemia and the need for newer prophylactic strategies against oxidative radicals in arteriogenic ED.

Ultrastructural damage such as loss of endothelium, mitochondrial injury and increased caveolae in our model are consistent with histological changes reported in human vasculogenic ED (Aydos et al., 1996; Grein & Schubert, 2002). Thickening and sporadic loss of erectile tissue endothelium have been documented in vasculogenic ED patients (Aydos et al., 1996). The role of caveolae in impaired erectile smooth muscle relaxation has been reported in rats (Yulia et al., 2009). Swollen vacuolated mitochondria and degradation of mitochondrial granules have been documented in erectile tissues from vasculogenic ED patients (Aydos et al., 1996). Factors leading to mitochondrial damage and eventually their disruption in penile ischaemia may relate to oxidative reactions within the mitochondrial elegant complexes I–V of energy production. It has been shown that a small portion of electrons passing through the mitochondrial electron transport chain, mostly at complexes I and III, react with molecular oxygen and produce $\text{O}_2^-$ (Blinova et al., 2008). The oxidatively strained mitochondria undergo deformation and turn to a source of free radicals and oxidative elements. This initiates a cascade of cellular and subcellular reactions in the surrounding tissues leading to smooth muscle dysfunction, structural damage and ultimately degeneration. Our finding that dietary antioxidants may protect mitochondrial structural integrity in penile ischaemia may be of great clinical implications.

Although basic research evidence of oxidative stress in ED is growing, clinical information is scarce, correlative and somewhat controversial. In the clinical setting, a possible correlation between ED and oxidative stress markers in blood samples has been reported, but free radical injury in dysfunctional erectile tissues from ED patients is yet to be documented. Several studies reported...
increased lipid peroxidation and reactive oxygen species and marked changes in antioxidant status in penile blood samples from ED patients (Fernández-Arjona et al., 2001; Tostes et al., 2008; Barassi et al., 2009). In contrast, a study comparing penile Doppler ultrasound findings with oxidative markers reported no significant correlation between tumescence grade or penile haemodynamic parameters and myeloperoxidase and malondialdehyde levels or SOD activity in the blood sample from corpus cavernosum of ED patients (Serefoglu et al., 2009). Confl-icting clinical reports may relate to correlative methodologies as circulating levels of oxidative stress markers in blood samples could originate from cardiovascular system or other organs and not necessarily from the penis. Newer technologies to detect free radical injury in penile erectile tissue and its vasculature may help better assessment of oxidative stress in ED patients.

In summary, our studies with the experimental model suggest the involvement of oxidative stress in arteriogenic ED. The mechanism may involve oxidative products resulting from lack of perfusion, antioxidant deficiency and accumulation of waste products. Oxidative radicals may contribute to impaired endothelium-dependent smooth muscle relaxation, mitochondrial injury, endothelial structural damage and erectile tissue fibrosis in arteriogenic ED. Upregulation of SOD and AR gene expression in arteriogenic ED may be an intrinsic defensive reaction against oxidative stress that apparently failed to protect erectile tissue against oxidative injury in our model. Antioxidant therapy appears to act promptly and more efficiently on the molecular and ultrastructural alterations of erectile tissue than on distinct pathological changes such as fibrosis and functional deficit. Our data suggest that long-term consumption of dietary antioxidants may remove oxidative products and improve erectile function, possibly by protecting NO bioavailability and preserving endothelial and mitochondrial structural integrity. Therapies targeting free radical generation and basic mitochondrial processes may lead to newer strategies against smooth muscle dysfunction and erectile tissue fibrosis in arteriogenic ED.

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