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**Early Intervention of Tyrosine Nitration Prevents Vaso-obliteration and
Neovascularization in Ischemic Retinopathy**

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Running Title:

Nitration of p85 and retinal vaso-obliteration

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Abbreviations: Bovine retinal endothelial (BRE) cells, peroxynitrite (PN), vascular endothelial growth factor (VEGF), poly-ADP-ribose polymerase (PARP), post-natal day (p), N-acetyl cysteine (NAC), Retinopathy of prematurity (ROP), Diabetic retinopathy (DR).

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Abstract

Diabetic retinopathy and retinopathy of prematurity are blinding disorders that follow a pathological pattern of ischemic retinopathy and affect premature infants and working age adults. Yet, the treatment options are limited to laser-photocoagulation. The goal of this study is to elucidate the molecular mechanism and examine the therapeutic effects of inhibiting tyrosine nitration on protecting early retinal vascular cell death and late neovascularization in ischemic retinopathy model. Ischemic retinopathy was developed by exposing neonatal mice to 75% oxygen (p7-p12) followed by normoxia 21% oxygen (p12-p17). Peroxynitrite decomposition catalyst FeTPPs (1 mg/Kg), the nitration inhibitor epicatechin (10 mg/Kg) or the thiol donor N-acetyl cysteine (NAC, 150 mg/Kg) were administered (p7-p12) or (p7-p17). Vascular endothelial cells were incubated at hyperoxia (40% oxygen) or normoxia (21% oxygen) for 48 hours. Vascular density was determined in retinal flat-mounts labeled with iso-lectin B4. Expression of VEGF, caspase-3 and PARP, Activation of Akt and p38 MAPK and tyrosine nitration of the PI3 kinase p85 subunit were analyzed by Western-blot. Hyperoxia-induced peroxynitrite caused endothelial cell apoptosis as indicated by expression of cleaved caspase-3 and PARP leading to vaso-obliteration. These effects were associated with significant tyrosine nitration of the p85-subunit of PI-3kinase, decreased Akt activation and enhanced p38 MAPK activation. Blocking tyrosine nitration of PI-3kinase with epicatechin or NAC restored Akt phosphorylation, and inhibited vaso-obliteration at p12 and neovascularization at p17 comparable to FeTPPs. Early inhibition of tyrosine nitration using epicatechin or NAC can represent safe and effective vascular protective agents in ischemic retinopathy.

Introduction

Retinopathy of prematurity (ROP) and diabetic retinopathy (DR) are potentially blinding disorders that affect premature infants and working age adults, respectively in US (Aiello et al., 1998; Chen and Smith, 2007). ROP and DR follow a pathological progression pattern characteristic of ischemic retinopathy, where the loss of retinal capillary is an early initiating event, leading to a poorly controlled process of retinal neovascularization and the development of proliferative retinopathy (for review see (Caldwell et al., 2003)). So far, the golden standard treatment for retinal neovascularization is limited to laser photocoagulation. Although successful, this treatment is invasive and results in loss of peripheral vision (for review see (Ali and El-Remessy, 2009)). The lack of approved pharmacological treatment for DR and ROP creates a great need for finding new effective therapeutic modalities to treat these devastating diseases. Accordingly, the mechanisms that control the process of retinal neovascularization are therefore of major clinical importance.

Peroxynitrite formed by reaction of nitric oxide and superoxide anion, mediates a variety of biological processes including inhibition of key metabolic enzymes, lipid peroxidation, nitration of the protein tyrosine residue and reduction of cellular antioxidant defenses by oxidation of thiol pools (Pacher et al., 2007). A critical role of increased oxidative stress and in particular the peroxynitrite is supported by previous studies showing that increases in peroxynitrite formation causes capillary endothelial cells apoptosis leading to vascular cell loss in DR and ROP models (Brooks et al., 2001; Gu et al., 2002; Sennlaub et al., 2002; Beauchamp et al., 2004; Kowluru and Odenbach, 2004; Kowluru et al., 2007). Moreover, clinical studies showed that infants with active ROP have low serum levels of reduced glutathione (GSH) suggesting compromised anti-

oxidant defense (Papp et al., 1999; Wright et al., 2006). We and others have shown that increased peroxynitrite formation correlates with capillary endothelial cells apoptosis (Du et al., 2002; Sennlaub et al., 2002; Zou et al., 2002; Gu et al., 2003; El-Remessy et al., 2005; Drel et al., 2008). However, the molecular mechanism for peroxynitrite-induced vascular cell death and the therapeutic role of blocking tyrosine nitration were not investigated in models of ischemic retinopathy.

In the present study, we took the advantage of using ischemic retinopathy mouse model as it has two distinguished stages: initial stage of hyperoxia 75% oxygen characterized with excessive peroxynitrite formation and capillary drop out followed by a later stage of hypoxia 21% oxygen characterized with mild oxidative insult and retinal neovascularization (Al-Shabrawey et al., 2005; El-Remessy et al., 2007). We compared the effects of selectively blocking the tyrosine nitration using epicatechin, one of the green tea extracts that has no antioxidant properties but selectively inhibits tyrosine nitration process, versus FeTPPs the specific peroxynitrite decomposition catalyst or N-Acetyl cysteine (NAC) which is a dietary supplement, general antioxidant and a thiol donor. While we showed previously that blocking tyrosine nitration using the selective nitration inhibitor epicatechin can restore VEGF survival signal (El-Remessy et al., 2005), it enhances VEGF's angiogenic signal *in vitro* and retinal neovascularization *in vivo* (El-Remessy et al., 2007).

Our previous studies showed that high levels of peroxynitrite inhibit prosurvival signal of VEGF and bFGF and cause endothelial cell death in culture models of diabetic retinopathy and retinopathy of prematurity (Gu et al., 2003; El-Remessy et al., 2005). Here, we elucidate the molecular mechanism by which peroxynitrite-mediated tyrosine nitration can inhibit p85 subunit

of PI 3-kinase and trigger vascular cell death *in vivo*. We also investigate the effects of the early intervention of tyrosine nitration on preventing retinal vascular cell death and vaso-obliteration during hyperoxic stage (p7-p12) and the continuous intervention (p7-p17) on preventing retinal neovascularization. Our results suggest that early blocking tyrosine nitration and peroxynitrite formation could be considered as a new effective therapeutic target for a possible control of common ischemic proliferative retinopathy diseases.

Materials and Methods

Cell culture

Primary cultures of bovine retinal endothelial (BRE) cells were prepared as described previously (El-Remessy et al., 2007). Cells from *passages 4–8* were used in all experiments. Cells were maintained in M199 supplemented with 10% FBS, 10% CS-C complete medium, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified CO₂ incubator.

Treatment during hyperoxia and normoxia: BRECs were grown to 80% confluence and then switched to serum-free medium and placed in a hyperoxic (40% O₂, 5% CO₂) or normoxic (21% O₂, 5% CO₂) environment for 48 h unless otherwise indicated. The 40% O₂ level was chosen for hyperoxia 40% oxygen exposure on the basis of previous research showing that 40% O₂ generates significant peroxynitrite without inducing toxicity in retinal cells (Gu et al., 2003). The hyperoxia 40% oxygen exposure was performed in a humidified incubator modified by installing the PROOX model 110 oxygen regulator (Biospherix, Redfield, NY). The oxygen level was continuously monitored using the PROOX oxygen sensor.

Animals

All experiments were performed using C57Bl/6 mice and were approved by the institutional Committee for Animal Use in Research and Education at the VA Medical center and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Ischemic retinopathy mouse model

Following the protocol of (Smith et al., 1994), on postnatal day 7 (p7) newborn mice were placed along with their dams into a custom-built chamber in which the partial pressure of oxygen

was maintained at 75% (Biospherix , Redfield, NY). Mice were maintained in 75% oxygen for up to 5 days (p12), after which they were transferred back to room air (relative hypoxia 21% oxygen). Room temperature was maintained at 20⁰C, and rooms were illuminated with standard fluorescent lighting on a 12-hour light–dark cycle. Newborn mice were nursed by the dams that were given food (standard mouse chow) and water.

Treatment during hyperoxia and hypoxia periods: Animals were treated by daily intraperitoneal injections (IP) with the following inhibitors: peroxy-nitrite decomposition catalyst 5, 10, 15, 20 tetrakis (4sulfonatophenyl) porphyrinato iron III chloride (FeTPPs, 1mg/Kg, Calbiochem), the nitration inhibitor, (–)-*cis*-3,3',4',5,7-Pentahydroxyflavane, (2*R*,3*R*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol, (epicatechin, 10mg/Kg, Sigma), or the general antioxidant, N-acetyl cysteine (NAC, 150mg/Kg Sigma). Animals were treated either during hyperoxia stage (75% oxygen) only (p7-p12) or during hyperoxia (75% oxygen) and hypoxia stage 21% oxygen (p7-p17). Control animals were injected with the vehicle. The treatment regimen was optimized and described before (El-Remessy et al., 2007).

Dissecting retinal tissue: Pups were deeply anesthetized by IP injection of Avertin 240 mg/Kg. One eye was enucleated and fixed in 2% PFA overnight to be flat- mounted. For the other eye, retinas were isolated and snap frozen for biochemical assays.

Analysis of Vaso-Obliteration and Neovascularization

Retinal vascular distribution was analyzed using retinal flat mounts labeled with biotinylated *Griffonia simplicifolia* lectin B4 and Texas Red–conjugated Avidin D (Vector Laboratories, Burlingame, CA). Retinas were viewed and imaged with fluorescence AxioObserver Zeiss Microscope (Germany). Vaso-obliteration was assessed on p12 and p17 as described previously

(Al-Shabrawey et al., 2005). The areas of retinal neovascularization were assessed on p17 as described previously (El-Remessy et al., 2007).

Oxidized and reduced Glutathione ratio

Total glutathione including reduced (GSH) and oxidized (GSSG) were measured using a kit (Northwest Life Science, Vancouver, WA) according to manufacturer's protocol. For total glutathione, retinas were lysed in phosphate buffer (100mM potassium phosphate and 1mM EDTA) and were mixed with an equal amount of DTNB (10 mM 5, 5'-dithiobis (2-nitrobenzoic acid) in the presence of glutathione reductase and NADPH producing a measurable yellow color. The color was measured at a wavelength of 412 nm. To detect GSSG, samples were treated with 10 mM 2-vinylpyridine (Sigma, St. Louis, MO) in ethanol to sequester all the reduced GSH then measured using the same protocol of the glutathione. GSH was calculated as the difference between total glutathione and GSSG.

Determination of retinal lipid peroxides

The assay was performed on retinal lysates as described before (Ali et al., 2008). Briefly, retinal lysate is reacted with 20% Acetic Acid, 8% SDS and thiobarbituric acid at 95°C for 60 min and the reaction was cooled down on ice. The samples were centrifuged and the supernatant was extracted with n-butanol and pyridine (15:1 respectively) and the absorbance of the organic solvent layer measured at 532 nm. The results were compared with an external standard (Tetramethoxypropane). The Bradford assay (Bio-Rad, Hercules, CA) was performed to determine the protein concentration of the retinal lysate. Lipid peroxide level was expressed in nmol MDA /mg total protein.

Western blotting analysis

Retinas and BREC were harvested after various treatments and lysed in modified RIPA buffer (Millipore, Billerica, MA) 30 min on ice. Insoluble material was removed by centrifugation at 14,000 *g* at 4°C for 30 min. 50 µg of total protein were boiled in 6x Laemmli sample buffer, separated on a 10–12% SDS-polyacrylamide gel by electrophoresis, transferred to nitrocellulose, and reacted with specific antibody. The primary antibodies for cleaved caspase-3, phosphor-p38, p38, phospho-Akt, or Akt were obtained from (Cell Signaling, Boston, MA) and were detected using a horseradish peroxidase-conjugated antibody and enhanced chemiluminescence (Amersham BioSciences). The films were subsequently scanned, and band intensity was quantified using densitometry software (Alpa Innotech). For PI 3-kinase tyrosine nitration, retinal lysates were incubated with p85 antibody and A/G agarose beads overnight. The precipitated proteins were analyzed by SDS-PAGE and blotted with nitrotyrosine antibody or p85 for equal loading as described above. Antibodies for p85 subunit of the PI-3 kinase and nitrotyrosine were obtained from (Millipore, Billerica, MA). For VEGF, retinal lysates were subjected to heparin beads (Sigma, St. Louis, MO) as described before (Platt et al., 2005). Briefly, the beads were pelleted at 5000 x *g* for 1 min, washed in 400 mM NaCl and 20 mM Tris and loaded onto a 4-20% gradient Tris glycine pre-cast gel (BioRad, Hercules, CA). After blocking, the membrane was incubated with VEGF primary antibody (Calbiocam, Gibbstown, NJ). The band was visualized and quantified as described above.

Detection of nitrotyrosine

Relative amounts of proteins nitrated on tyrosine were measured using slot-blot techniques as described previously (El-Remessy et al., 2003). Briefly, RIPA lysate were immobilized onto nitrocellulose membrane by using a slot blot microfiltration unit (Bio-Rad, Hercules, CA). A dilution series of peroxynitrite-modified BSA (Cayman Chemical, Ann Harper, MI) was loaded to generate a standard curve, and nitrotyrosine was detected using a polyclonal anti-nitrotyrosine antibody (Millipore, Temecula, CA) followed by peroxidase-labeled goat anti-mouse IgG and ECL. Relative levels of nitrotyrosine immunoreactivity were determined by densitometry software (Alpa Innotech).

Data Analysis

The results were expressed as mean \pm SE. Difference among experimental groups were evaluated by analysis of variance, and the significance of difference between groups was assessed by the post-hoc test (Fisher's PLSD) when indicated. Significance was defined as $P < 0.05$.

Results

Blocking tyrosine nitration decreases retinal vaso-obliteration

Previous studies showed a positive correlation between retinal vascular cell death, increases in peroxynitrite formation and tyrosine nitration. Therefore, we screened the protective effects of selective inhibition of tyrosine nitration using epicatechin or the general antioxidant and the thiol donor N-acetyl cysteine (NAC) versus decomposing peroxynitrite using (FeTPPs), a selective peroxynitrite decomposition catalyst. As shown in (Fig. 1.A-D), exposing the developing retina of pups (p7-p12) to high oxygen concentration caused retinal vaso-obliteration as indicated by the capillary drop out area in the central retina. Treatment of pups with FeTPPs (1mg/Kg), epicatechin (10mg/Kg), or NAC (150mg/Kg) significantly reduced central capillary dropout with 34.2%, 25%, 42% respectively (Fig. 1.E). NAC showed superior effect over epicatechin in protecting the retina from vascular cell death as shown (Fig. 1.E). Treatment of control pups with FeTPPs, epicatechin, or NAC did not alter physiologic retinal vascular density (Data not shown). These results suggest that selective blocking of tyrosine nitration has protective effect comparable to decomposing peroxynitrite or preventing thiol oxidation on retinal vascular cell death.

Blocking tyrosine nitration prevents retinal apoptosis

Apoptosis has been postulated as the mechanism by which vascular cell death occurs in ischemic retinopathy. Therefore, we investigated the expression of apoptotic markers prior to p12. Exposing the retina (p7-p9) to high oxygen resulted in increases in cleaved caspase-3 (Fig. 2.A). Treatment with FeTPPs, epicatechin, or NAC significantly reduced cleaved caspase-3 with 46.5%, 51%, 43.5% respectively (Fig. 2.A). Apoptosis was further confirmed by detecting poly-ADP-ribose polymerase (PARP), a cleavage target of caspase-3 (Fig. 2.B). Retinas exposed to

hyperoxia (75% oxygen) showed an increase in PARP activation. Treatment with FeTPPs, epicatechin, or NAC reduced PARP activation with 50%, 64%, 71.5% respectively.

Effect of inhibition of tyrosine nitration on VEGF

Down-regulation of VEGF expression has been postulated to cause vaso-obliteration of the newly formed capillaries (Alon et al., 1995). Therefore, we tested whether the protective effects of blocking tyrosine nitration on preventing retinal vaso-obliteration involve alteration of VEGF expression. As shown in Fig. 3, exposing the retina (p7-p12) to high oxygen resulted in modest but significant decreases in VEGF expression compared to retinas developed at normal oxygen. Interestingly, treatment with FeTPPs, epicatechin, or NAC did not affect VEGF expression compared to PBS-treated pups (Fig. 3).

Blocking tyrosine nitration selectively decreases nitrate stress

The retina is believed to be vulnerable to nitrate and oxidative damage because of the abundance of polyunsaturated fatty acids. Hyperoxia (75% oxygen) showed a significant increase in the nitrotyrosine formation compared to normoxia (21% oxygen) (Fig. 4.A). Treatment of pups with FeTPPs, epicatechin, or NAC significantly decreased nitrotyrosine formation (50%, 58% and 60% respectively). In addition to tyrosine nitration, peroxynitrite can cause thiol oxidation which compromises antioxidant defense. Measuring the ratio between the cellular oxidized to reduced glutathione is used as a marker for the retinal antioxidant defense. Normal tissue has a balanced ratio between oxidized and reduced glutathione. As shown in (Fig. 4.B) Retinas from p12 pups exposed to high oxygen showed (4-fold) increases in tissue GSSG/GSH ratio. Treatment with either FeTPPs or NAC significantly restored the GSSG/GSH

ratio and increased the retinal antioxidant defense. In contrast, treatment with epicatechin has a modest but not significant effect on GSSG/GSH ratio.

Hyperoxia causes tyrosine nitration of p85 subunit of PI 3-kinase

Our previous work has shown that the p85 regulatory subunit of PI 3-kinase is a susceptible target for peroxynitrite-induced tyrosine nitration in endothelial cells (El-Remessy et al., 2005). As shown in (Fig 5.A), retinas from p12 that were exposed to high oxygen showed a (4-fold) increase in tyrosine nitration of p85 compared to normoxia (21% oxygen). Treatment (p7-p12) with epicatechin or NAC significantly reduced the nitrating effect of peroxynitrite on tyrosine residues similar to decomposing peroxynitrite with FeTPPs.

Hyperoxia-induced nitration inhibits Akt survival and activates p38 MAP kinase

As the retina is composed of several cell types, we further examined the role of tyrosine nitration in hyperoxia-induced vascular cell death. Bovine retinal endothelial (BRE) cells were cultured in high oxygen conditions (40%) and compared to normoxia (21%). BRE cells showed 2.3-fold increase in tyrosine nitration of the p85 subunit compared to normoxia (21% oxygen) (Fig. 5.B). Treatment with FeTPPs (2.5 μ M), epicatechin (100 μ M) or NAC (1 mM) for 48 hrs significantly reduced hyperoxia-induced p85 tyrosine nitration (Fig. 5.B). To confirm the inhibitory effect of tyrosine nitration on PI3-Kinase, we tested the effects of inhibitors on Akt phosphorylation. Akt, a down stream target of PI3-Kinase, mediates cell survival by inhibiting apoptotic processes. Hyperoxia (40% oxygen) significantly decreased Akt phosphorylation compared to normoxia (21% oxygen) by 40 % (Fig 6.A). Treating cells with FeTPPs, epicatechin or NAC for 48 hrs restored Akt activation. PI 3-kinase/Akt signaling promotes endothelial cell survival by inhibiting p38 MAPK-dependent apoptosis (Gratton et al., 2001). Therefore, blockade of PI 3-

kinase/Akt via tyrosine nitration of p85 subunit can lead to enhanced activation of p38 MAPK and endothelial apoptosis. As shown in Fig. 6B, cells cultured in hyperoxia (40% oxygen) showed 2-fold in p38 MAPK phosphorylation that was decreased by treatment with FeTPPs, epicatechin or NAC. Increases in p38 MAPK phosphorylation were also observed in p12 retinas subjected to high oxygen compared to controls (Fig 6.C). Retinas from PBS-treated pups showed 2-fold increase in p38 MAPK phosphorylation compared to normoxia (21% oxygen). Treatments with FeTPPs, epicatechin or NAC reduced p38 MAPK phosphorylation by 34.6%, 36.6% and 49.1% respectively. Next, we evaluated the effects of hyperoxia (40% oxygen) to induce endothelial cell apoptosis. As shown in Fig. 6.D, hyperoxia (40% oxygen) induced endothelial cell apoptosis as indicated by increased expression of cleaved caspase-3. Blocking tyrosine nitration with FeTPPs, epicatechin or NAC protected the cells against apoptosis by decreasing the level of cleaved caspase-3 by 50%, 43% and 46% respectively.

Early intervention of tyrosine nitration prevents retinal oxidative injury.

Lipid peroxidation has been established as a general marker of oxidative stress and cellular injury. As shown in Fig. 7A, retinas from pups that underwent hyperoxia (75% oxygen) and hypoxia 21% oxygen (p7-p17) showed high levels of lipid peroxides compared to the one in normal air. Early intervention with FeTPPs, epicatechin or the thiol donor NAC decreased lipid peroxidation hence the cellular injury. Interestingly, treatment with FeTPPs or the thiol donor NAC, but not epicatechin restored the antioxidant defense as indicated by the ratio of GSSG to GSH (Fig. 7.B). These results confirm the effect of epicatechin in selectively blocking tyrosine nitration without exerting antioxidant effect.

Early intervention of tyrosine nitration prevents retinal neovascularization

Our previous studies showed that blocking nitration after vaso-obliteration (p12-p17) did not prevent retinal neovascularization (El-Remessy et al., 2007). Here we tested the long-term protective effects of blocking tyrosine nitration during both hyperoxia (75% oxygen) and hypoxia 21% oxygen (p7-p17). Compared to PBS-treated pups, treatment with epicatechin significantly (62.1%) reduced retinal neovascularization. This effect was comparable but to lesser extent to pups treated with FeTPPs or NAC by 76.8% and 71.5% respective reduction in neovascularization (Fig. 8.A-E).

Next, we measured the capillary drop out areas at the end of hypoxia 21% oxygen (p17) to test the effect of treatment on the physiological revascularization in the central retina. As expected in this model, PBS-treated controls showed a 29% reduction in capillary drop out areas compared to the one at the end of hyperoxia (75% oxygen) (p12). Treatment with FeTPPs, epicatechin or NAC further reduced capillary drop out areas by 55%, 50%, and 60% respectively compared to p17 PBS-treated animals; suggesting that blocking peroxynitrite did not alter physiological revascularization; instead, it enhanced vascular regrowth and continued protecting from vaso-obliteration (Fig 8.F).

Discussion

The present study documents novel data suggesting that 1) Peroxynitrite mediates retinal vaso-obliteration via tyrosine nitration of p85 kinase and inhibition of PI 3kinase/Akt survival pathway; 2) Blocking tyrosine nitration restores survival signal and prevents endothelial cell apoptosis and retinal ischemia and 3) early intervention with treatments that target peroxynitrite and tyrosine nitration prevents retinal neovascularization. To our knowledge this is the first *in vivo* study to elucidate the mechanism by which peroxynitrite-mediated tyrosine nitration and inhibition of PI 3-kinase survival pathway trigger vascular cell death in ischemic retinopathy model. Our results also demonstrate that targeting tyrosine nitration with dietary supplements such as epicatechin and NAC represent potentially safe and effective therapeutic strategy that could be translated to patients with ischemic-proliferative diseases.

The focus of the current study is to test the protective effects of blocking peroxynitrite and tyrosine nitration on retinal cell death *in vitro* and on preventing vaso-obliteration and neovascularization *in vivo*. Therefore, we took the advantage of using ischemic retinopathy mouse model as it has two distinguished stages: initial vascular cell death and vaso-obliteration, which are associated with excessive peroxynitrite formation followed by retinal neovascularization which is associated with mild oxidative insult (Al-Shabrawey et al., 2005; El-Remessy et al., 2007). We used epicatechin, a flavonoid and one of the green tea extract that selectively block peroxynitrite-mediated tyrosine nitration but not thiol oxidation (Schroeder et al., 2001; El-Remessy et al., 2005; El-Remessy et al., 2007) and compared its effects to blocking peroxynitrite using FeTPPs, the peroxynitrite decomposition catalyst or blocking thiol oxidation using the dietary supplement and the thiol donor NAC (El-Remessy et al., 2005; El-Remessy et

al., 2007). Our results showed that exposing the developing retina to high oxygen induces significant capillary drop out areas that was accompanied with nitrotyrosine formation and apoptosis as indicated by increased expression of cleaved caspase-3 expression and poly (ADP-ribose) polymerase (PARP). These results are in agreements with previous experimental and clinical studies showing that ischemic retinopathy is associated with increases in retinal oxidative damage and decreases in antioxidant defense (Papp et al., 1999; Brooks et al., 2001; Gu et al., 2002; Beauchamp et al., 2004; Wright et al., 2006; Bartoli et al., 2008). Our results showing that hyperoxia caused significant increases in cleaved PARP, the caspase-3 substrate *in vitro* and *in vivo* lend further support to the previous reports of the critical role of caspase-3 in executing peroxynitrite-induced apoptosis in endothelial cells (Kotamraju et al., 2001; Gu et al., 2003; Zhu et al., 2004; El-Remessy et al., 2005). Treatment with epicatechin blocked tyrosine nitration and significantly reduced capillary dropout areas and the expression of cleaved caspase-3 and PARP. However, epicatechin did not alter GSSG/GSH ratio confirming its selective properties in blocking tyrosine nitration but not thiol oxidation. On the other hand, treatment with FeTPPs or NAC not only blocked tyrosine nitration but also improved GSSG/GSH ratio indicating restoration of retinal antioxidant defense which explain the superior effects of NAC and FeTPPs in reducing vaso-oblivation over epicatechin. In contrast, NAC failed to show protective effects on reducing retinal avascularity and apoptosis in a rat model of ROP that could be attributed to the different nature of model and shorter duration of treatment (Saito et al., 2007). Our results establish a unique role of selective blocking tyrosine nitration, apart from the antioxidant effect, in preventing retinal vaso-oblivation and support the notion of its therapeutic intervention for ischemic retinal diseases.

To elucidate the molecular mechanisms by which blocking tyrosine nitration protect the retina from apoptosis, we determined the expression of VEGF as its down-regulation has been postulated to cause vaso-obliteration of the newly formed capillaries (Alon et al., 1995). In agreement, our results showed modest but significant decrease in VEGF expression in hyperoxic retinas. However, treatment with FeTPPs, epicatechin or NAC did not alter VEGF expression compared to PBS-treated ones. These results suggest that the protective effects of blocking peroxynitrite and tyrosine nitration in preventing retinal apoptosis and vaso-obliteration are not mediated by altering VEGF expression level, instead by modulating VEGF signal.

VEGF's function as a survival factor for endothelial cells is well established (Duh and Aiello, 1999). VEGF activation of VEGFR2 transduces anti-apoptotic signal via PI 3-kinase/Akt signaling pathway (Gerber et al., 1998; Fujio and Walsh, 1999). Tyrosine nitration and subsequent inhibition of the p85 regulatory subunit of PI 3-kinase have been documented in response to peroxynitrite (Hellberg et al., 1998; El-Remessy et al., 2005). Here, we show that retinas from pups exposed to hyperoxia (75% oxygen) showed significant increases (4-fold) in tyrosine nitration of p85 subunit compared to pups in normoxia (21% oxygen). Similar pattern was observed in retinal endothelial cultures maintained at hyperoxia (75% oxygen) compared to the one maintained at normal oxygen. These effects were associated with impaired Akt phosphorylation, increased p38 MAPK phosphorylation and increased expression of the apoptotic marker caspase-3. Treatment with epicatechin or NAC had comparable effects to FeTPPs, and prevented tyrosine nitration of p85, restored Akt survival pathway and reduced the activation of p38 MAPK apoptotic pathway. These results confirm the relationship between nitration of p85, decreases in Akt activity and the pro-apoptotic effects of hyperoxia-induced

peroxynitrite. Our findings lend further support to previous reports of significant increases in oxidative and nitrative stress in ischemic retinopathy model (Papp et al., 1999; Brooks et al., 2001; Gu et al., 2002; Gu et al., 2003; Beauchamp et al., 2004; Saito et al., 2007; Medina et al., 2008). Understanding of the molecular pathogenesis of ischemic retinopathy provides the basis for identifying novel therapeutic targets. The role of the hypoxia induced factors VEGF and erythropoietin as well as the maternally-derived factors insulin-like growth factor-1 have begun to be elucidated (see review (Heidary et al., 2009)). However, our study is the first we know of that elucidates the molecular mechanism of the tyrosine nitration of p85 leading to inactivation of, the PI 3-kinase/Akt survival signal and activation of the pro-apoptotic p38 MAPK signal in ischemic retinopathy model. A scheme of the proposed mechanism is depicted in Fig. 9. These findings explain the protective effects of epicatechin in preventing vaso-obliteration even though it did not completely restore retinal anti-oxidant defense.

Retinal neovascularization takes place as result of initial stage of capillary loss leading to inner retinal ischemia that drives up-regulation of angiogenic growth factors (Mizutani et al., 1996). The combined vascular protection of reducing pathological neovascularization while allowing physiological revascularization of the retina is the ideal therapeutic modality for ischemic retinopathy diseases. Therefore, it is critical to evaluate the effects of early blocking of tyrosine nitration and capillary drop out on preventing the subsequent neovascularization. Continuous intervention of tyrosine nitration during both hyperoxia (75% oxygen) and hypoxia 21% oxygen (p7-p17) with epicatechin, FeTPPs or NAC significantly reduced lipid peroxidation, retinal neovascularization and further reduced central capillary drop out compared to PBS-controls. The vascular protective effects of epicatechin in the current study are mainly due its effects in

preventing earlier vaso-obliteration and hence the stimulus to neovascularization. This concept is further supported by our previous findings that late intervention with epicatechin after vaso-obliteration (p12-p17) did not prevent retinal neovascularization (El-Remessy et al., 2005; El-Remessy et al., 2007). The vascular protective effects of NAC and FeTPPs were associated with restoration of anti-oxidant defense as indicated by GSSG/GSH ratio, but not of epicatechin confirming its selective effect of inhibiting tyrosine nitration apart from thiol oxidation. While the vascular protective effects of FeTPPs are significant, the fact that it contains iron will limit its therapeutic use for chronic administration. The vascular effects of NAC were superior to epicatechin but did not reach significance and this could be attributed to the antioxidant of NAC over epicatechin. New studies showed that NAC also may have other mechanism that can interfere with cell proliferation by regulating the cell cycle regulatory protein (Menon et al., 2007). However, both epicatechin and NAC prove effective and the fact that they are already available as dietary supplement open the door for therapeutic utility. Together, these results point out the importance of the timing and the molecular target of intervention. In support, studies using the same ischemic retinopathy model showed that early intervention with omega-3 polyunsaturated fatty acid failed to rescue oxygen-induced vessel loss during hyperoxia (75% oxygen) but prevented late retinal neovascularization (Connor et al., 2007). On the other hand, intervention with statins effectively reduced retinal oxidative stress, capillary drop out and prevented neovascularization (Bartoli et al., 2008; Medina et al., 2008).

In conclusion, our study is the first to elucidate the mechanism by which tyrosine nitration of p85 kinase and inhibition of PI 3-kinase/Akt survival pathway cause vascular cell death *in vivo* and to demonstrate the vascular protective effects of the early and continuous intervention of

peroxynitrite and tyrosine nitration on retinal vessel loss and neovascularization. Furthermore, our results provide compelling evidence that targeting tyrosine nitration with safe dietary supplements such as epicatechin and NAC in animal models should provide the rationale for testing these agents as a possible control of common ischemic proliferative retinopathy such as DR and ROP.

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Footnotes

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Legends for Figures

Fig. 1. Blocking peroxynitrite formation or tyrosine nitration decreases retinal vaso-obliteration.

(A-D) Representative images of flat-mounted retinas labeled with GSI lectin to examine vaso-obliteration. Mice pups were maintained in hyperoxia (75% oxygen) (p7-p12) and treated with FeTPPs (1 mg/kg/day, i.p.), epicatechin (10 mg/kg/day, i.p) or NAC (150 mg/kg/day, i.p.). Exposing the developing retina to high oxygen resulted in 35% central capillary dropout area (Shaded yellow areas). Treatment with FeTPPs, epicatechin or NAC significantly reduced the capillary dropout areas.

(E) Statistical analysis of the ratio of central capillary dropout areas to the total retinal area in PBS-treated and other treated retinas showing the protective effects of blocking peroxynitrite formation and tyrosine nitration on retinal vaso-obliteration. (n=10-12, *P<0.05, VS PBS-treated p12; #P<0.05, VS NAC).

Fig. 2. Blocking peroxynitrite formation or tyrosine nitration prevents retinal apoptotic markers.

Representative image of Western blot analysis of retinal lysates (50 µg) (A) Exposing retinas to hyperoxia (75% oxygen) (p7-p9) significantly increased cleaved caspase-3 expression compared to normoxic control. Treatment with FeTPPs (1 mg/kg/day, i.p.), epicatechin (10 mg/kg/day, i.p) Exposing retina to hyperoxia (75% oxygen) (p7-p9) resulted in significant increases in apoptosis as indicated by 2 fold increase in cleaved PARP expression compared to normoxic control. Treatment with FeTPPs (1 mg/kg/day, i.p.), epicatechin (10 mg/kg/day, i.p) or NAC (150

mg/kg/day, i.p.) normalized cleaved PARP expression (C) Statistical analysis of the ratio of cleaved caspase-3 or PARP expression to actin ($n=4$, $*P<0.05$, VS control).

Fig. 3. Effect of inhibition of tyrosine nitration on VEGF expression.

A representative image for Western blot analysis of retinal lysates (100 μ g) shows that exposing retina to hyperoxia (75% oxygen) (p7-p12) significantly decreased retinal VEGF expression compared to normal oxygen control. Treatment with FeTPPs (1 mg/kg/day, i.p.), epicatechin (10 mg/kg/day, i.p) or NAC (150 mg/kg/day, i.p.) did not alter VEGF expression ($n=4$, $*P<0.05$, VS control).

Fig. 4. Blocking tyrosine nitration selectively decreases nitrate stress but not antioxidant defense.

(A) A representative image for slot blot analysis of retinal lysates (30 μ g) shows that exposing retina to hyperoxia (75% oxygen)(p7-p12) significantly increases retinal nitrotyrosine formations. Treatment with FeTPPs (1 mg/kg/day, i.p.), epicatechin (10 mg/kg/day, i.p) or NAC (150 mg/kg/day, i.p.) significantly decreased tyrosine nitration. ($n=4$, $*P<0.05$, VS control).

(B) Statistical analysis of the ratio of oxidized to reduced glutathione (GSSG/GSH) in different retinal lysate. Hyperoxia (75% oxygen) (p7-p12) increased GSSG/GSH ratio 4-fold compared to normoxia (21% oxygen). Treatment with FeTPPs (1 mg/kg/day, i.p.) or NAC (150 mg/kg/day, i.p.) restored the retinal antioxidant defense back to normal. Epicatechin (10 mg/kg/day, i.p) did not alter GSSG/GSH ratio. ($n=4$, $*P<0.05$, VS control).

Fig. 5. Hyperoxia causes tyrosine nitration of p85 subunit of PI 3-kinase.

- (A) Immunoprecipitation with anti-p85 subunit of the PI 3-kinase and Western blot analysis using anti-nitrotyrosine antibody show that exposing retina to hyperoxia (75% oxygen) (p7-p12) significantly increased nitration on the regulatory p85 subunit compared with normal retinas. This effect was reduced by treatment with FeTPPs (1 mg/kg/day, i.p.), epicatechin (10 mg/kg/day, i.p) or NAC (150 mg/kg/day, i.p.) ($n=4$, $*P<0.05$, VS control, # $P<0.05$, VS PBS-treated p12).
- (B) Immunoprecipitation with anti-p85 subunit of PI 3-kinase and Western blot analysis using anti-nitrotyrosine antibody showed that cells cultured in high oxygen conditions (40%), significantly increased nitration of the regulatory p85 subunit compared with cells cultured in normal oxygen. This effect was blocked by the specific peroxynitrite decomposition catalyst FeTPPs (2.5 μM) and the specific nitration inhibitor epicatechin (100 μM) and thiol donor NAC (1 mM). ($n=4$, $*P<0.05$, VS normoxia 21% oxygen)

Fig .6.Hyperoxia-induced nitration inhibits Akt survival and activates p38 MAP kinase.

Western Blot analysis of (50 μg) lysates from retina or cells that were cultured in high oxygen conditions (40%) and compared to normoxia (20%). (A) Hyperoxia (40% oxygen) decreased the Akt phosphorelation by 40%. Treating cells with FeTPPs (2.5 μM), epicatechin (100 μM) or NAC (1 mM) for 48 hrs restored Akt activity. ($n=4$, $*P<0.05$, VS normoxia 21% oxygen).

(B) Hyperoxia (40% oxygen) increased the p38 phosphorylation with 2 fold increase. Treating cells with FeTPPs (2.5 μM), epicatechin (100 μM) or NAC (1 mM) for 48 hrs decreased p38 phosphorelation. ($n=4$, $*P<0.05$, VS normoxia 21% oxygen). (C) Exposing retina to hyperoxia (75% oxygen) (p7-p12) significantly increases p38 MAPK phosphorylation compared to normoxia (21% oxygen). Retinas from PBS-treated pups

showed 2-fold increase p38 MAPK phosphorylation compared to normoxia (21% oxygen). Treatment with FeTPPs (1 mg/kg/day, i.p.), epicatechin (10 mg/kg/day, i.p) or NAC (150 mg/kg/day, i.p.) reduced p38 MAPK phosphorylation by 34.6%, 36.6% and 49.1% respectively. ($n=4$, $*P<0.05$, VS control). **(D)** Hyperoxia (40% oxygen) significantly increases cellular apoptosis as indicated by 1.5 fold increase in cleaved caspase-3 expression compared to normoxic control. Treatment with FeTPPs (2.5 μ M), NAC (1 mM) or epicatechin (100 μ M) for 48 hrs reduced cleaved caspase-3 expression to normal levels. ($n=4$, $*P<0.05$, VS normoxia 21% oxygen).

Fig. 7. Early intervention of tyrosine nitration prevents retinal oxidative stress

(A) Statistical analysis of lipid peroxidation showing that exposing neonates to hyperoxia (75% oxygen) then hypoxia 21% oxygen (p7-p17) increased retinal cellular injury indicated by significant increases in lipid peroxidation. Treatment with FeTPPs (1 mg/kg/day, i.p), epicatechin (10 mg/kg/day, i.p) or NAC (150 mg/kg/day, i.p.) decreased it. ($n=8$, $*P<0.05$, VS control).

(B) Statistical analysis of the ratio of oxidized to reduced glutathione (GSSG/GSH) showing that hyperoxia (75% oxygen) followed by hypoxia 21% oxygen (p7-p17) significantly increased the retinal GSSG/GSH ratio (3-fold. Treatment with FeTPPs (1 mg/kg/day, i.p.) or NAC (150 mg/kg/day, i.p.) restored the normal antioxidant defense. Epicatechin (10 mg/kg/day, i.p) did not alter GSSG/GSH ratio. ($n=8$, $*P<0.05$, VS control).

Fig. 8. Early intervention prevents retinal neovascularization

(A-D) Representative images of flat-mounted retinas labeled with GSI lectin to examine retinal neovascularization. Mice pups were maintained in hyperoxia (75% oxygen) followed by hypoxia

21% oxygen (p7-p17) which resulted in neovascularization as indicated by tufts in the peripheral retinas. Treatment with FeTPPs (1 mg/kg/day, i.p.), epicatechin (10 mg/kg/day, i.p) or NAC (150 mg/kg/day, i.p.) significantly reduced the neovascularization areas.

(E) Statistical analysis of the ratio of neovascularization areas to total retinal showing the protective effects of blocking peroxynitrite formation and tyrosine nitration on retinal neovascularization. ($n=8$, $*P<0.05$, VS p17 PBS-treated).

(F) Statistical analysis of the ratio of central capillary dropout areas to total retinal area in (p7-p17) treated retinas showing that treatment with FeTPPs (1 mg/kg/day, i.p.), epicatechin (10 mg/kg/day, i.p) or NAC (150 mg/kg/day, i.p.) significantly reduced capillary dropout with 54%, 50%, 60% respectively. ($n=8$, $*P<0.05$, VS p17 PBS-treated).

Fig. 9. A schematic representation of the proposed mechanism by which high oxygen via peroxynitrite inactivates VEGF/ PI 3-kinase/Akt-1 pro-survival pathway and stimulates cell death via activation of p38 MAP kinase pathway. Nitration of PI 3-Kinase is proposed as a mechanism by which peroxynitrite switches off the VEGF pro-survival and triggers the proapoptotic path

Fig. 1

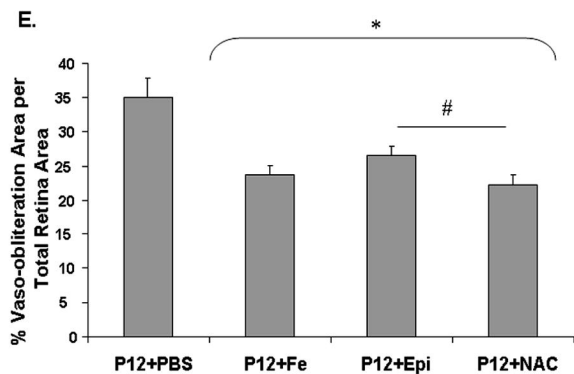
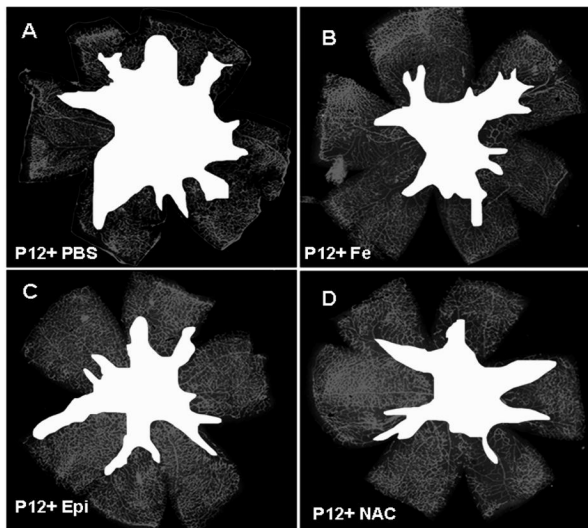
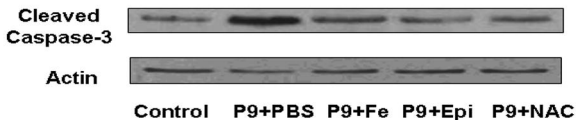
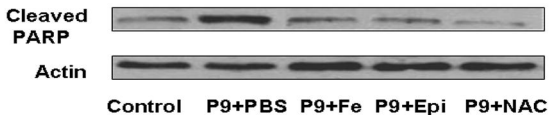


Fig. 2

A



B.



C.

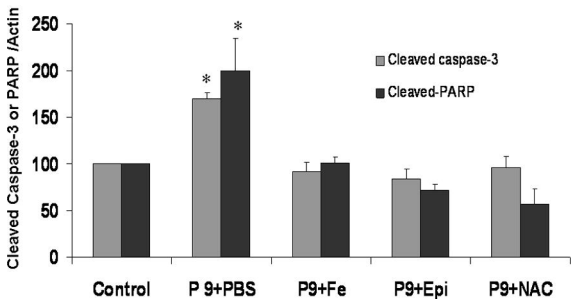


Fig. 3

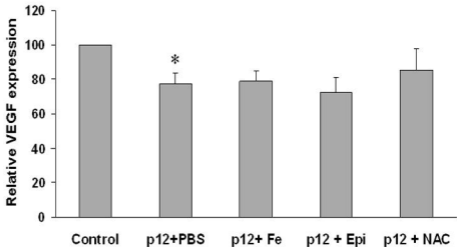
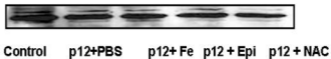
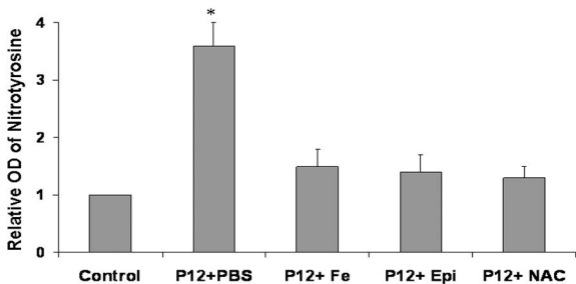
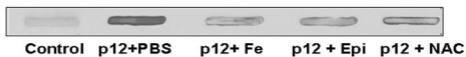


Fig. 4

A.



B.

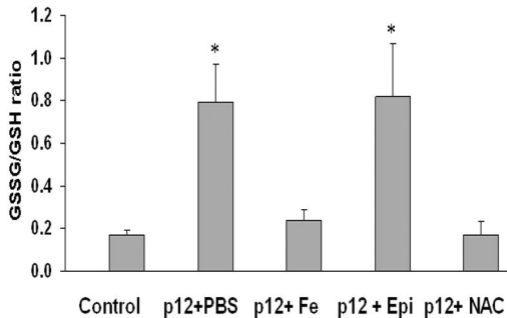
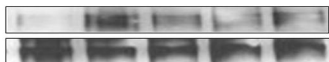


Fig. 5

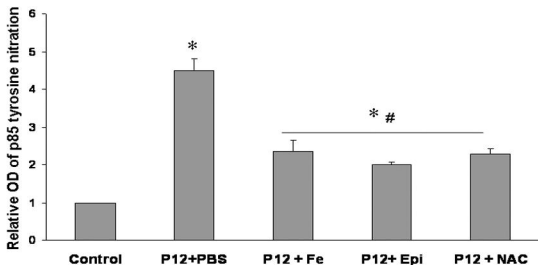
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I.B. NY

I.B. P85



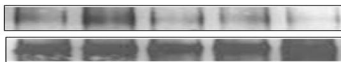
Control p12+PBS p12+Fe p12+Epi p12+NAC

A.**B.**

I.P. P85

I.B. NY

I.B. P85



Normoxia Hyperoxia H+ Fe H+ Epi H+ NAC

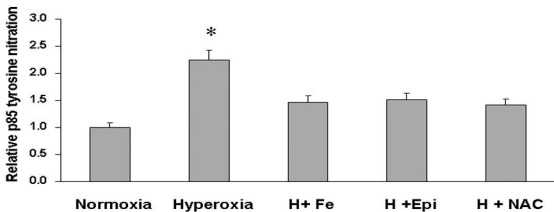


Fig. 6

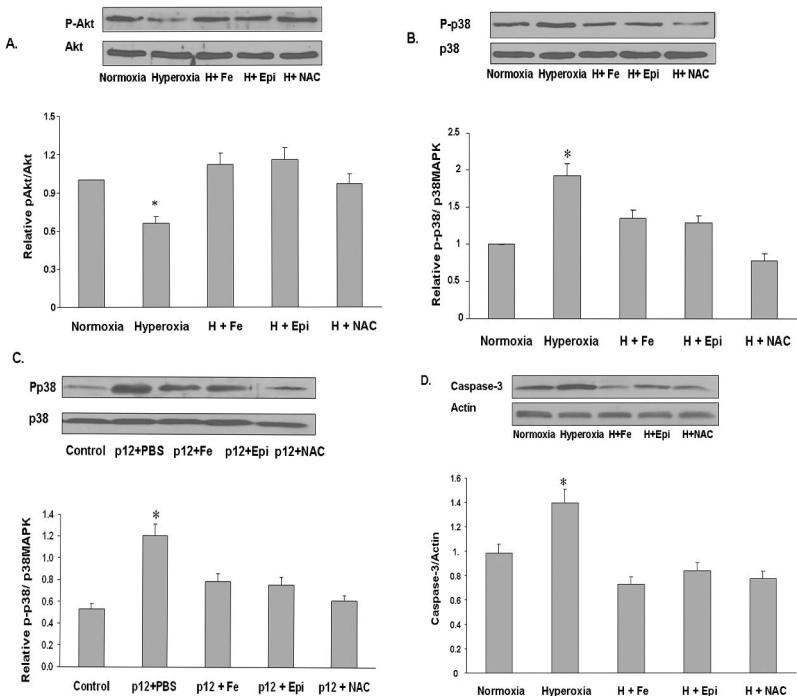
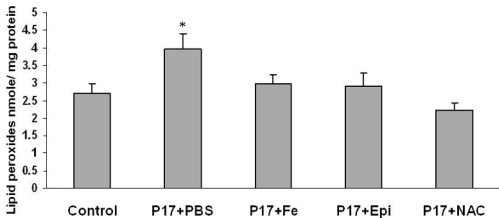


Fig. 7

A.



B.

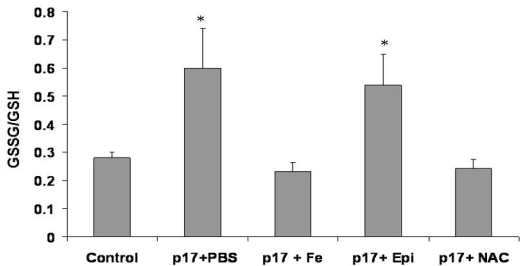


Fig. 8

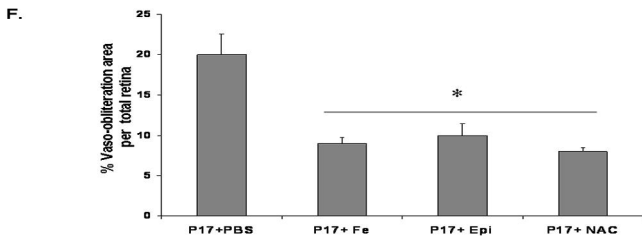
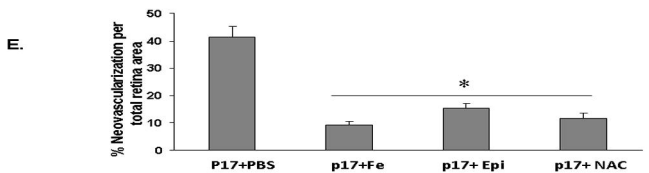
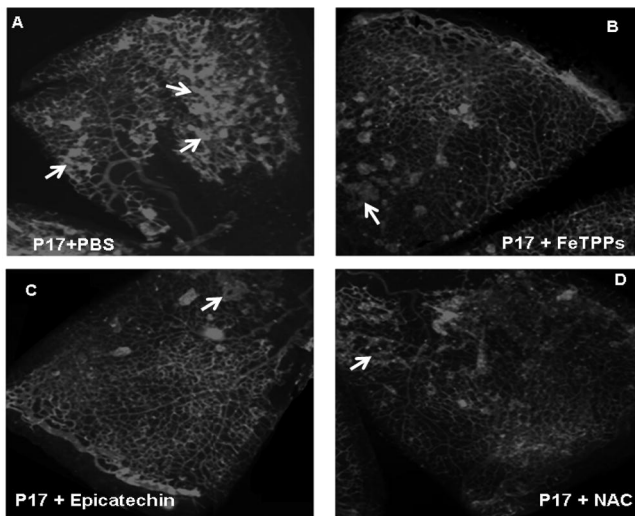


Fig. 9

