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Hypoxia perturbs endothelium by re-organizing cellular actin architecture: Nitric oxide offers limited protection

Short running title: NO protects Hypoxia induced actin pattern in ECs

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Highlights

- Hypoxia induces alteration in actin filaments in chick embryo extra vascular model and endothelial cells
- Hypoxia induces paracellular gaps in blood capillaries of chick embryo extra vascular model.
- NO confers protection against hypoxia-mediated cytoskeltal remodeling and endothelial leakiness

Abstract

Exposure to hypoxia causes structural changes in the endothelial cell (EC) monolayer that alter its permeability. There was a report earlier of impairment of nitric oxide (NO) production in endothelium. Intervention of NO in the altered cellular arrangements of actin cytoskeleton in endothelium for rectification of paracellular gaps in endothelium under hypoxia was observed. The present study demonstrates hypoxia inducing paracellular gaps in hypoxia exposed blood capillaries in chick embryo extra vascular model. Phalloidin staining confirmed significant polymerization of actin and unique cellular localization of the F-actin bands under hypoxia treatments. Addition of spermine NONOate (SPNO), a NO donor, or reoxygenation to endothelial monolayer attenuated hypoxia-mediated effects on endothelial permeability with

partial recovery of endothelial integrity through actin remodeling. The present study indicates link of hypoxia-induced actin-associated cytoskeletal rearrangements and paracellular gaps in the endothelium with a low NO availability in the hypoxia milieu. The author concludes that NO confers protection against hypoxia-mediated cytoskeletal remodeling and endothelial leakiness.

List of Abbreviations: cGMP - cyclic guanosine monophosphate; DAPI - (4',6-Diamidino-2-Phenylindole, Dihydrochloride;; EC - endothelial cell; ECs - endothelial cells; eNOS - endothelial nitric oxide synthase; FBS - fetal bovine serum; FITC - Fluorescein isothiocyanate; HAPE - high-altitude pulmonary edema; HIF 1α - hypoxia inducible factor 1 alpha; L-NAME - L-N^G-Nitroarginine methyl ester; MRTF - myocardin-related transcription factor; MRTF-A - myocardin-related transcription factor A; MRTF-B - myocardin-related transcription factor B; NO - Nitric Oxide; PBS - phosphate buffer saline; ROS - reactive oxygen species; SPNO - Spermine NoNoate; SRF- serum response factor; TRITC - Tetramethylrhodamine; TMBH2O2 - 3,3′,5,5′-Tetramethylbenzidine hydrogen peroxide.

Keywords: Actin filaments; Endothelium; Gap junction; Hypoxia; Nitric Oxide; Permeability.

1. Introduction

Hypoxia extenuates nitric oxide (NO) is produced in endothelial cell and destabilizes vascular homeostasis. Low oxygen level in blood induces morphological alteration in the endothelial monolayer (**Hackett and Roach, 2004**) followed by disturbed endothelial integrity,

increased permeability and resulting eventually in endothelial barrier dysfunction (**Bärtsch et al., 2005; Hackett and Roach, 2004; Wojciak-Stothard B et al., 2006; Aslam M et al., 2013; Seerapu et al., 2010**). There have been reports lately of hypoxia making alterations in the cellular actin dynamics through changes made in the cell shape and adhesion (**Misra et al., 2012; Volgar et al., 2013**). The formation of polarized actin band in the cellular loci under hypoxia speeds up physical tension at cell-to-cell interface, with contribution to the formation of paracellular gaps in the monolayer (**Kolluru et al., 2007**). Alteration in the vascular permeability of the ECs in vessel wall through changes in the actin pattern as a result of NO alteration has been demonstrated (**Baldwin et al., 1998; Fischer et al., 1999**). Inducement of barrier dysfunction from hypoxia mediated attenuation by Rho-kinase dependent actin modulation in the EC is obvious (**Parikh et al., 2012; Wojciak-Stothard et al., 2012**).

Inhalation of NO improves oxygenation in hypoxemic patients and offers protection from hypoxia-induced endothelial leakiness by NO/cGMP pathways (**Teman et al., 2015; Kolluru et al., 2007**). **Palmer et al. (1987) and Michelakis et al. (2002)** report that NO obviates chances of lungs getting leaky during any injury apart from maintaining vascular integrity in the respiratory system. Inhalation of NO was used for the treatment of high-altitude pulmonary edema (HAPE) (**Mundy and Dorrington, 2000; Michelakis et al., 2002; Himashree et al., 2003; Perrin et al., 2006**). **Liu et al. (1997)** report that NO and cGMP regulating the permeability and actin filament in reactive oxygen species (ROS) stressed ECs.

The author has reported earlier that NO-cGMP analogue rescues endothelium from hypoxia-induced vascular leakiness under reduced oxygen milieu (**Kolluru et al., 2007**). The work now presented establishes inducement of leakiness by hypoxia in the vasculature of chick embryo area vasculose, while exogenous supplementation of NO partially makes a revision in

hypoxia induced leakiness. The study further emphasizes on hypoxia mediated actin dependent endothelial remodeling, with eventual proof of the leakiness in the blood vessels.

2. Materials and Methods

2.1 Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from PAN-Biotech (Aidenbach,Germany). Spermine NoNoate was purchased from CAYMAN chemicals(Michigan,USA). DAPI, Phalloidin-Alexa Fluor 568 (phalloidin), Rhodamine Phalloidin, and Texas Red were purchased from Invitrogen Life Technologies. β – Actin and HIF-1 α antibodies were purchased from Abcam (Kolkata, India). All other chemicals were reagent grade.

2.2 Cell line, culture conditions and animals

Immortalized endothelial cell lines, EAhy926, a benign gift from Dr. C.J.S. Edgell, Mayo Clinic (Rochester, Minn., U.S.A.), were used for our study (Edgell et al., 1983). The endothelial cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin–streptomycin (w/v) and maintained in a 5% CO_2 humidified incubator at 37ºC.

Fertilized brown leghorn chicken eggs were purchased from Government poultry station, Potheri, Chennai, India and incubated in sterile humidified incubator at 37°C. All the experimental manipulations in chick embryos were performed on the 4th day of incubation.

2.3 Hypoxia treatment in cell model

ECs were cultured in a 12-well plate and allowed to grow until reaching 100% confluence. The culture plate was placed into a special chamber system with an inlet and an outlet for purging the required percentage of O_2. For the control sets, the cells were incubated under normoxia

conditions. For all the hypoxia experiments, a gas mixture (5% O_2 + 95% N_2) was purged in the hypoxia chamber for 1 h (**Seerapu et al., 2010**)

2.4 Hypoxia treatment of chick vascular bed

2.4.1 Global hypoxia

The fertilized egg was opened in sterile dishes on the fourth day. The chick vascular bed contained dish was placed in the hypoxic chamber. Then a gas mixture ((5% O_2 + 95% N_2) was purged in the hypoxia chamber. The temperature in the hypoxic chamber was maintained at 37°C for 2 h. For control sets, the vascular beds were maintained at 37°C, 5% CO_2 for 2 h.

2.4.2 Creating ischemic on chick vascular bed using right vitelline artery ligation model

The fertilized egg was opened in sterile dishes on the fourth day. The chick right vitelline artery was blocked using surgical suture creating ischemic condition and kept at 37°C for 2 h. For control sets, the vascular bed contained dish was kept at 37°C for 2 h (**Majumder et al., 2010**).

2.5 Alexa Fluor-phalloidin staining in chick vascular bed

The chick vascular beds were treated with global hypoxia and ischemic for 2 h. Next, the isolated vascular beds from egg yolk were washed with 1X PBS. The vascular beds were fixed with 4% of paraformaldehyde and permeabilized with 0.1% of Triton X. Finally, they were stained with Alexa Fluor-phalloidin (10 µM) and incubated for 10 min. The vascular beds were then washed with 1X PBS. Images were captured at 20X magnification using Olympus IX71 epifluorescence microscopy system equipped with a DP71 camera.

2.6 Live cell imaging of cell morphology under hypoxia

ECs cultured on glass cover slips, were placed in the lower chamber of live cell hypoxia chamber. The lower chamber was then closed with upper chamber of live cell hypoxia chamber. Preheated water was circulated in and around the live cell chamber for maintaining the

temperature at 37°C. The hypoxic gas mixture was purged into the chamber. Cells were observed through a glass slide attached to the upper chamber. Images were taken from 0 to 80 min and analysed on the basis of cell morphology.

2.7 Phalloidin Rhodamine staining in ECs

ECs cultured on glass cover slips were treated under hypoxia and normoxia. Then the cells were fixed, permeabilized, and incubated with 10µM Palladian-Rhodamine at 37ºC for 20 min. Fluorescent images of actin filaments were observed for pattern changes during hypoxia induction (**Kolluru et al., 2007**).

2.8 Permeability assay in chick vascular bed

The vascular bed was carefully separated from egg yolk without any damage, placed on a fresh dish and washed with 1X PBS to remove the excess egg yolk. The vascular beds were treated with SPNO (Spermine-NONOate) and without SPNO and placed in the hypoxic chamber for 2 h at 37°C. For purpose of control, the vascular beds were treated with SPNO and without SPNO for 2 h at 37 °C and 5% CO_2. After treatment, the vascular beds were gently washed with PBS, then incubated with 10 µM of Texas Red for 30 min.The vascular beds were fixed with 2% of paraformaldehyde for 10 min.

2.9 Amperometric measurement of oxygen using an oxygen sensitive electrode

Oxygen measurement was carried out on ECs grown on cover slips with the aid of an amperometric probe (Apollo 4000 analyzers, WPI Inc., Sarasota, Fla., U.S.A.) as described earlier (**Veeriah et al., 2015**). ECs were seeded on cover slips upto 100% confluence and were treated under hypoxia (5% O_2 at 37°C) for 30 min and 1 h. In order to ensure control, cells were kept at 37°C and 5% CO_2. Following hypoxia treatment, the EC monolayer was washed and immersed in 1ml of PBS. The electrode was located in a glass well and the top of the electrode

was placed 1mm above the surface of the cells and observed by a real-time acquisition of oxygen level through a single control panel computer that exhibits the experimental data.

2.10 Immunofluorescence

ECs were seeded in 24 well plates treated with 10 µM of SPNO. ECs were kept in normoxia and hypoxia condition for 1 h. Fixing and permeabilizing of the cells were done as described elsewhere (**Mukhopadhyay et al., 2007**), after which they where incubated overnight at 4ºC with anti-rabbit α-tubulin primary antibody (dilution 1:1000) and anti-mouse F-actin primary antibody (1:1000 diltion) combined together and added to the cells. This was followed by the addition of goat anti-rabbit IgG-TRITC (dilution 1:2000) and goat anti-mouse IgG-FITC (dilution 1:2000) to the cells coupled to FITC and incubated for 1 h at room temperature. The nucleus was stained with DAPI.

2.11 Western blot

The protein samples were boiled in Lamelli Buffer for 5 min at 90ºC prior to loading on 10% SDS-PAGE (**Nakashima et al., 1999**). Following electrophoresis, the proteins were transferred onto nitrocellulose membrane (MDI, 0.45 micron) using a wet blotting apparatus (Medox, India). Transfer of proteins onto the membrane was done by staining with ponceau S. Following the transfer, the membranes were blocked using 5% non-fat milk and probed for respective primary antibodies (1:1000) and peroxidase labeled secondary antibodies (1:2000). The blots were developed using TMB/H_2O_2 substrate from Bangalore Gene, India. Verification of molecular weights of the protein was done by comparing with a broad range protein marker ranging from 10 kDa to 250 kDa.

2.12 Statistical Analysis

All the experiments were performed in triplicate (n=3) unless otherwise specified. Data, that was analyzed using the one-way ANOVA test, Student t-test, and the Tukey post hoc test, as appropriate. Data is presented as mean + SE. P values smaller or equal to 0.05 were used as the criteria for statistically significant difference.

3. Results

3.1 Hypoxia induces changes in endothelium via actin remodeling in chick vascular bed

A study of low oxygen effect on chick vascular endothelial integrity was made through use of a partial ischemia model of chick embryo area vasculosa by blocking the right vitelline artery of chick vascular bed (**Fig. 1A**). The vascular bed was fixed after a 2 h treatment and stained with phalloidin-FITC. The group with ischemic treatment appeared leaky compared to the normoxic control (**Fig. 1B**). However, the group with global hypoxia treatment showed a large number of paracellular gaps. An intact monolayer was observed in the case of normoxic blood vessels (**Fig. 1C**). The number of gaps was calculated from the images and plotted the graph. The graph represents that number of gaps was significantly increased in global hypoxia and partial ischemic treated chick vascular bed compred to normoxia treated group (**Fig. 1D**). The author quantified the expression of hypoxia induced HIF-1α expression in ECs by Western blot and found hypoxia upregulating HIF-1α expression (**Supplementary Fig. 1A**). The levels of oxygen in hypoxia treated endothelial monlolayer were measured and compared with normoxia using an ultra sensitive oxygen electrode (**Supplementary Fig. 1B**). The result demonstrated increases in oxygen level under 1 h hypoxia treated ECs compare to normoxia and 30 min hypoxia treated ECs.

3.2 Study of real-time effects of hypoxia on ECs morphology and actin polymerization using live cell hypoxia chamber

Hypoxia induces cytoskeletal rearrangements that contribute to morphological changes in ECs (**Kayyali et al., 2002**). Hypoxia-mediated actin remodeling at adherent junctions in ECs creates gaps (both transcellular and paracellular gaps) at cell-cell interface contributing to leakiness in endothelial monolayer (**Liu et al., 2009**). Construction of live cell hypoxia chamber information provided in supplementary material (**Supplementary Fig. 2**) EC was exposed to hypoxia and the images were captured at different time points (0 to 80 min) using the live cell hypoxia

chamber. This was done for the purpose of monitoring the live morphological changes that occur in it. Result indicate the hypoxia exposure made the cells rounded at 60 min (**Fig. 2**).

3.3 Hypoxia mediates alteration in cytoskeletal patterns and cell-cell interaction in ECs

ECs were seeded on collagen-coated cover glasses and treated for hypoxia and normoxia for 1 h at 37ºC for the purpose of observing the effect of hypoxia on the actin cytoskeleton and cell- to-cell interaction. ECs were stained with phalloidin fluorescent probe for visualizing F-actin filament organization. Results indicate the presence of a significant polymerization of F-actin bands under hypoxia treatment that give an alteration in the cell shape (**Fig. 3A**). The white arrows in fig. 3A denote the F-actin filament organization in normoxia and hypoxia treated ECs. The cell- to- cell interaction was disturbed under hypoxia treated ECs to normoxia treated ECs (**Fig. 3B**). The white arrows in fig. 3B indicate the paracellular gaps that have appeared in hypoxia condition. These data suggest the contribution of hypoxia to the paracellular gap formation and thereby it may contribute causing of ECs monolayer leakiness

3.4 Quatification of actin rearrangements of ECs under hypoxic condition

The authour hypothesized that hypoxia improvises changes in endothelial monolayer by altering cellular actin polymerization and thereby cell-cell interactions. The ECs were treated under hypoxia and normoxia for the investigation of the role of hypoxia in causing characteristic cell shapes associated with cellular actin polymerization. They were then processed for phalloidin staining and analyzed by fluorescent imaging. The cells were categorized strictly on the basis of the cellular actin polymerization as evident from phalloidin staining pattern. Next, distribution pattern of the sub-population was quantified. The sub-populations were termed as cobblestone, caged, nested and armor in fig. 4A. Results indicate the maintenance of a steady state ratio under

normoxia by the population distribution pattern of the cells while there was a drastic change in hypoxia (**Fig. 4A**). Hypoxia treatment significantly reduced the number of cobblestone cells and elevated the number of caged and nested cells (**Fig. 4B**). This panel illustrate the global effect of hypoxia induced sub-population distribution pattern in ECs (**Fig. 4C**). The inference was that the actin filaments gradually polymerize at the periphery and finally causing the rounding up of cells.

3.5 Reoxygenation recovers ECs from hypoxia-mediated morphological changes

Experiments done earlier suggest the responsibility of hypoxia-mediated actin rearrangements for alterations in the cell shape. Hypoxia followed by reoxygenation is highly deleterious to the cell system due to the outburst of ROS (**Therade-Matharan S et al., 2005**). However, the general recovery mechanism after hypoxia involves the resupply of oxygen for regaining its normal functions. In order to study the effect of hypoxia followed by reoxygenation, fluorescence imaging of actin was performed in ECs treated for hypoxia and reoxygenation at different duration ranging from 15 min to 90 min. The results indicate changes in actin patterns under hypoxia compared to normoxia. The phalloidin imaging data suggest that reoxygenation helps the ECs to gain partial recovery from hypoxia-mediated morphological changes conferred due to actin rearrangements. Parallel live cell imaging experiments were performed to check the effect of reoxygenation in the EC morphological status (**Fig. 5A**). The graph in (**Fig. 5B**) represents the percentage of rounded cells under hypoxia/reoxygenation treatments followed upto 120 min. The data suggests that return of oxygen to the hypoxia-treated cells showing 38% recovery from hypoxia-mediated alterations in cell morphology.

3.6 NO protects cellular actin polymerization pattern of the ECs under hypoxia

ECs were seeded on the coverslip, and kept at 37°C and 5% CO_2 overnight. The cells were treated with SPNO and without SPNO and then placed in the hypoxic chamber for 30 min, 1 h, and 2 h. To ensure, control cells were treated with SPNO and without SPNO, and kept at 37°C and 5% CO_2. Then cells were fixed and stained with Alexa Fluor Phalloidin. The images of Alexa Fluor-phalloidin staining also demonstrated the cell to cell morphological integrity under hypoxia, and SPNO (NO) implications in the normalization of hypoxia-mediated alterations in the cell to cell interactions (**Fig. 6**). The panel shows a strong actin polymerization pattern at the interface of two ECs under normoxic and SPNO treatment at 0 h. The upper panel shows hypoxia treatment inducing thinning of polymerized actin bands at the interfaces and promotes more lamellipodia along with the intensity of hypoxia. Donation of NO from SPNO protects polymerized actin bands at the cell-cell interface and reduces the occurrence of lamellipodia compared to hypoxia treatment (**Fig. 6**).

3.7 NO protects the F-actin polymerization in ECs under hypoxia

ECs were treated with SPNO under normoxia and hypoxia conditions. Immunofluroscence experiment was then performed to check the F-actin and alpha tubulin expression under hypoxia. Since the polymerization of G-actin is directly proportional to alpha tubulin, it denotes the dynamics of G-actin and F-actin relationship at a given time. The results indicate hypoxia treated cells having more polymerized actin at the periphery of ECs, and stronger alpha tubulin pattern around the nucleus. Therefore, SPNO treated cells show smaller flurosecnce intensity of green (F-actin) and red (alpha tubulin) to hypoxia treated cells (**Fig. 7**). Data suggest partial protection for the polymerization of G-actin in ECs under hypoxia treatement from SPNO.

3.8 NO protects permeability of chick vascular blood vessel under hypoxia

Right vitelline artery was blocked for study the protective role of NO in vascular permeability and creating an ischemic condition. The ischemic condition of vascular bed was treated with SPNO and incubated at 37°C for 2 h. For control experiments, the vascular beds were treated with SPNO and without SPNO. The vascular bed was kept in a similar condition. The vascular bed was stained with Texas red (**Fig. 8A**). The Texas red dye intensity was calculated and plotted the graph. Results indicated the formation of paracellular gaps and creation of vascular leakiness under hypoxia. The possibility of donation of SPNO (a NO donor) preventing hypoxia- mediated leakiness in the vessels was observed. The panel exhibited a large permeation of Texas red dye into the blood vessels treated with hypoxia compared to normoxia and SPNO treated vessels. The addition of SPNO to hypoxic vessels showed that NO blocking hypoxia-mediated entry of Texas red dye into the blood vessels compared to an ischemic treated blood vessel. Donating NO was protecting the formation of the paracellular gap and reducing the permeation of Texas red dye into the blood vessel compare to an ischemic treated blood vessel (**Fig. 8A**).

3.9 NO protects endothelial integrity of chick vascular bed under hypoxia

For purpose of verification of the NO implications in cellular integrity of chick extraembryonic vascular bed under hypoxic condition, the vascular bed was treated with or without SPNO for 2 h. Next, the treated capillaries were fixed and stained with phalloidin FITC (**Fig. 8B**). The results demonstrated inducement of paracellular gaps by hypoxia, with the ability of SPNO treatment to block the slacking (**Fig. 8B**). The number of gaps was calculated from the images and plotted the graph. The graph represents that number of gaps was significantly increased in hypoxia treated chick vascular bed compred to normoxia treated group. The number of gaps was significantly decreased in hypoxia+SPNO treated chick vascular bed compared to hypoxia treated group.

4. Discussion

The arrangement of the cytoskeleton is an indispensable requirement for any eukaryotic cell requiring regulation from many cellular and physiological processes. Actin contributes to cellular shaping, motility, the polarity and cell division by dynamic processes of polymerization and de-polymerization. It is also involved in the maintenance of multicellular tissue organization, including the maintenance of cellular barrier function (**Pollard and Cooper, 2009**). Hypoxia has detrimental effects in various pathological conditions associated with hypoxia-mediated dysregulation of the actin cytoskeleton (**Zieseniss, 2014**). However, there has rather been poor understanding of morphological and phenotypical consequences of hypoxia mediated alterations of cellular actin dynamics in relation to NO in endothelium are poorly understood. The study presented here demonstrates hypoxia improvising cellular shape and architecture through changes in the cellular pattern of polymerized actin leads to the formation of paracellular and transcellular gaps in endothelium and NO reverses partially the hypoxia mediated changes in endothelium.

Studies made by the author show an increase in paracellular gaps in chick vascular bed under hypoxia treatment (**Fig. 1B**). An earlier report from our group suggests that leakiness of the endothelial bed is attributed to hypoxia exposures (**Kolluru et al., 2007**). The EC was probed with phalloidin fluorescent probe for the purpose of visualizing F-actin filament organization in endothelium. Results indicate that hypoxia treatment, following significant polymerization of F-actin bands, conferred an alteration in the cell shape compare to that of normoxia (**Fig. 3A, B**). Exposure of hypoxia to ECs causes a shift in filamentous actin from the web like structure to parallel stress fibers (**Kayyali et al., 2002**). In the present study, the author has made critical analysis of the subcellular pattern of polymerized actin in relation hypoxia treatments of

endothelial cells (**Fig. 4A, B**). The inference is that low oxygen environment triggers a signal for securing cell-to-cell interface in endothelium by enhancing and polarizing the pattern of actin polymerization since a continuous monolayer as the most important criterion for maintaining endothelial functions. Critical monitoring of the spatial distribution of polymerized actin, led to the observation of actin filaments as localized mostly in the cytoplasm for providing a basal shape and polarity of the cells under normoxic conditions, while hypoxia causes drastic reorganization of the cytoplasmic actin network, pushing stress fibers to the periphery of the cells (**Fig. 4A**). EC was treated with SPNO under normoxia and hypoxia respectively for . determining the intrinsic physiological implication of F-actin and G-actin. Immunofluroscence result indicates the ability of SPNO to partially prevent the hypoxia mediated polymerization in the endothelium (**Fig. 7**). These observations indicate that hypoxia re-formats intrinsic polarity of the cytoskeletal components by changing the course of cellular actin polymerization of endothelial cells.

It is evident that hypoxia down-regulates eNOS in endothelium (**Coulet et al., 2003**). Hypoxia-driven activation of hypoxia inducible factor-2 decreases in eNOS mRNA level via Rho kinase pathway in human venous and pulmonary artery ECs (**Coulet et al., 2003**). Increases in hypoxia arginase II activity in ECs which degrades l-arginine is also evident as an essential substrate for NO production by eNOS (**Clarkson et al., 2005**). Reduced NO availability results in endothelial dysfunction under hypoxia and thereby increases the risk for vascular diseases (**Atkeson and Jelic, 2008**). An experiment has been performed to study NO effect on the chick vascular bed under hypoxia condition particularly when NO is a critical regulator in vascular homeostasis. The Texas red permeability experiement indicate NO protection from hypoxia-mediated lekiness of endothelium and diminution of paracellular gaps in the endothelium monolayer (**Fig. 8A, B**).

There is also the observation of the ability of the ectopic release of NO from a NO donor to the ischemic blood vessel to partially block hypoxia-mediated entry of Texas red dye into the blood vessels compare to that of ischemic vessels (**Fig. 8A**). However, NO failed to recover the hypoxia-induced leakiness of endothelium completely. The limited recovery of endothelium leakiness by NO under hypoxia is explained this way; either the hypoxia assault overwhelmed the microenvironment beyond the repairing capability of NO signaling or hypoxic microenvironment uses other signaling pathways as well as to bring about the endothelial leakiness (**Fig. 9**). We postulate that hypoxia-induced re-formatting of cellular actin pattern triggers a series of signaling cascades, which ultimately helps endothelium adapting a low oxygen condition by altering structural and functional aspects of the cells. Hypoxia activates Rho-kinase and downregulate the eNOS expression, which ultimately disturbs the dynamics of cytoskeletal arrangements in endothelial cells (**Takemoto et al., 2002**). It has been shown that NO uses cGMP and subsequent Ca^{2+}/calmodulin to regulate actin reorganization in RAW 264.7 cells **(Ke et al., 2001).** Further, the work of **Lorenzo et al. (2013)** demonstrated that the loss of NO increased the recruitment of the Rac guanine-nucleotide-exchange factor (GEF) TIAM1 to adherens junctions and VE-cadherin in endothelial cells, and reduced Rho activation and stress fiber formation, which is actively linked with actin polymerization status of the cells. The work of **Olson and Nordheim (2010)** demonstrates rearrangement of the actin cytoskeleton, globular actin polymerization liberating myocardin-related transcription factor (MRTF) and its cofactors, thereby inducing the nuclear transcription factor serum response factor (SRF) for modulating the expression of genes encoding structural and regulatory effects of actin dynamics. Use of an inducible EC-specific deficiency of SRF model **Weinl et al. (2013)** showed the critical role

played by MRTF-A/MRTF-B in defining endothelial structure and protrusion in the mouse retinal primary vascular plexus.

NO implications in endothelial permeability often appears to be debatable, particularly in animal models. **Hinder et al. (1997)** employed 12 sheep chronically instrumented with lung lymph fistulas and hydraulic pulmonary venous occluders followed by the infusion of Escherichia coli endotoxin for 32 h. Permeability analysis at 32 h by venous occlusion techniques showed elevated filtration coefficients, while reversal by L-NAME of the systemic vasodilation during endotoxemia was associated with high pulmonary vascular resistance without evidence of impaired pulmonary endothelial barrier function . The final outcome of NO implications in endothelial permeability is defined by multi-factors such as NO dynamics, redox status of the microm-milieu, vascular maturity and other patho-physiological parameters. The present work elaborates turning of sub-cellualr dynamics of cytoskeleton by NO, and specifically F-actin polymerization to confer protection against hypoxia mediated alterations in cytoskeleton re-arrangements, cell-cell interface and endothelial leakiness.

4.1 Conclusion

The present study reveals inducement of polymerization of F-actin bands by hypoxia, and alters the cellular pattern of polymerized actin in the ECs and chick embryo vascular bed, respectively. This could be the reason for hypoxia mediated endothelial leakiness. Further, the present study confirms the protection to the endothelium leaky and improvzing chanages in actin dynamics under hypoxia milieu by ectopic release of NO.

Authors contribution

AS prepared the manuscript, designed, performed the experiments and analyzed the data, SC and GS Supervised, designed the experiments and approved the manuscript, DK, UB, and GK performed the experiments. All authors reviewed the manuscript.

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Conflict of Interest

There is no conflict of interest

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Figure Legends

Fig.1 Effect of low oxygen on endothelial integrity in Chick vascular blood vessel

1A. Schematic representation of how partial ischemia was created in chick vascular blood vessel **1B.** The global hypoxia, partial ischemic and normoxia treated capillaries were isolated and processed for actin staining. The group of ischemic treated blood capillaries showed paracellular gaps (marked in white arrow) compare to normoxic capillaries. The white arrows in the normoxia panel showed the intact monolayer of cells were observed (n=3). **1C.** The global hypoxia treated blood capillaries showed a large number of paracellular gaps compared to normoxic blood capillaries (marked in white arrow) (n=3). **1D.** Graph represents that number of gaps was calculated per filed of view of chick vascular bed treated with normoxia, partial ischemic and global hypoxia. **P<0.001 versus normoxia; *P =0.045 versus normoxia; n=3.Values represent the mean for each group SEM (one way ANOVA and LSD).

Fig. 2 Live cell imaging of EC morphology under hypoxic condition

2. The panel represents the time lapse image of the endothelial monolayer at 10 min interval up to 1.20 h under hypoxic condition. The arrows indicate changes in the cell morphology at different time points. Cells were almost got rounded up by 60 min time point (n=3).

Fig. 3 Effect of hypoxia on actin cytoskeletal pattern and cell- cell interaction in EC

3A. Effect of hypoxia on the cytoskeletal patterns in EC was studied using phalloidin staining. EC was treated for hypoxia and normoxia conditions, fixed, permeabilized and stained for actin with phalloidin. The changes in actin filament bands were observed by capturing images using

fluorescence microscope at 60X magnification. The panel shows images of EC treated for hypoxia and normoxia conditions. The white arrows indicate prominent thick bands of actin formed under hypoxia treatments (n=3). **3B.** EC cultured on collagen-coated cover glasses were treated for normoxia and hypoxia conditions. Next, the cells were fixed and processed for phalloidin staining. The white arrows indicate the paracellular gaps that widen during hypoxia (n=3).

Fig. 4 Effect of hypoxia on actin rearrangements in quantitative level

4A. Effect of hypoxia on actin rearrangements that yield different subpopulations in EC was studied using phalloidin staining. The cells were analyzed for different subpopulations 0n the basis of the rearrangements of actin filaments. The panel denotes different subpopulation depending on their actin band patterns. Subpopulations were classified into cobblestone, caged, nested and armor shapes. The panel shows images of EC treated for hypoxia and normoxia conditions. **4B.** The cell number of each subpopulation was counted and analyzed by plotting a graph. The graph represents the population distribution of four prominent cell shapes caused under normoxia and hypoxia treatments.Values represent means for each group ± SEM (**p < 0.001vs. normoxia, normoxia nested; ¥ p=0.02 vs normoxia caged; one-way ANOVA and LSD; n =3). This figure panel depicts that the global effect of hypoxia induced sub-population distribution pattern in ECs. The white arrow indicates that the five different sub population of ECs of hypoxia treated group (**Fig. 4C**).

Fig. 5: Effect of hypoxia and reoxygenation on EC morphology

5A. Panel A shows the image of representative morphology with a prominent actin pattern at a particular time point of hypoxia/reoxygenation. **5B.** Live cell experiments were performed using the live cell chamber for confirming the recovery of cell shapes. The cells were cultured on the collagen-coated coverslips and subjected to hypoxia treatments. The hypoxia-treated cells were taken out from hypoxia chamber and were placed in the live cell chamber they were then monitored for live cell imaging using phase contrast microscope with a DPI camera. Images were captured using at different time points at different fields and are analyzed by plotting a graph for number of rounded cells The data in the graph shows 40% recovery from hypoxia-mediated alterations in the EC morphology, Values represent means for each group ± SEM (* $p < 0.05$ vs normoxia; one-way ANOVA and LSD; n =3).

Fig. 6 Effect of NO on the cellular actin polymerization pattern of the EC under hypoxia

6. The images of Alexa Fluor-phalloidin staining also demonstrated the cell to cell morphological integrity under hypoxia, and SPNO (NO) implications in the normalization of hypoxia-mediated alterations in the cell to cell interactions. The upper panel shows that hypoxia treatment induces thinning of actin bands and promotes more lamellipodia compare to SPNO treated in hypoxia-treated endothelial cells (n=3).

Fig. 7 F-actin and G-actin expression in ECs under hypoxia treatment

7. Representative images of F-actin (green), Alpha tubulin (red) stained ECs cells treated with SPNO under normoxia and hypoxia condition n=3. Images were taken using fluorescence microscope. Graph respresents the F-actin and alpha tubulin fluorescence intensity were increased under hypoxia treatment compare to normoxia. ECs treated with SPNO under hypoxia

condition decreased the fluorescence intensity of F-actin and tubulin. Values represent means for each group ± SEM (**p < 0.001vs. normoxia, * p< 0.003 vs normoxia and hypoxia one-way ANOVA and LSD; n =3).

Fig. 8 Effect of NO on permeability and actin polymerization in chick embryo vascular blood vessel

8A. The result of the Texas red permeability assay showed the extent of dye permeation (vessel outline depicted with white arrows) in normoxia, hypoxia, SPNO and hypoxia with SPNO treated blood vessels. The graph indicates that the dye permeation was significantly increased in hypoxic treated chick vascular bed compared to normoxic treated group. The dye permeation was significantly decreased in hypoxia+SPNO treated with chick vacular bed compared to hypoxic treated group. Values represent means for each group ± SEM (**p < 0.001vs. normoxia and hypoxia one-way ANOVA and LSD; n =3).

.**8B.** The blood capillaries treated with normoxia, hypoxia, SPNO and hypoxia with SPNO for 2hr. The blood capillaries were fixed and stained with phalloidin conjugated with FITC. The red arrows indicate that paracellular gaps in hypoxia-treated capillaries compare to normoxia. Donating NO from SPNO partially recovered the intact structure of actin (n=3). The graph represents that number of gaps was significantly increased in hypoxia treated chick vascular bed compred to normoxia treated group. The number of gaps was significantly decreased in hypoxia+SPNO treated chick vascular bed compared to hypoxia treated group. . Values represent means for each group ± SEM (**p < 0.001vs. normoxia and hypoxia one-way ANOVA and LSD; n =3).

Fig. 9 Hypoxia perturbs the actin architecture: Suggested possible Rectification Strategy.

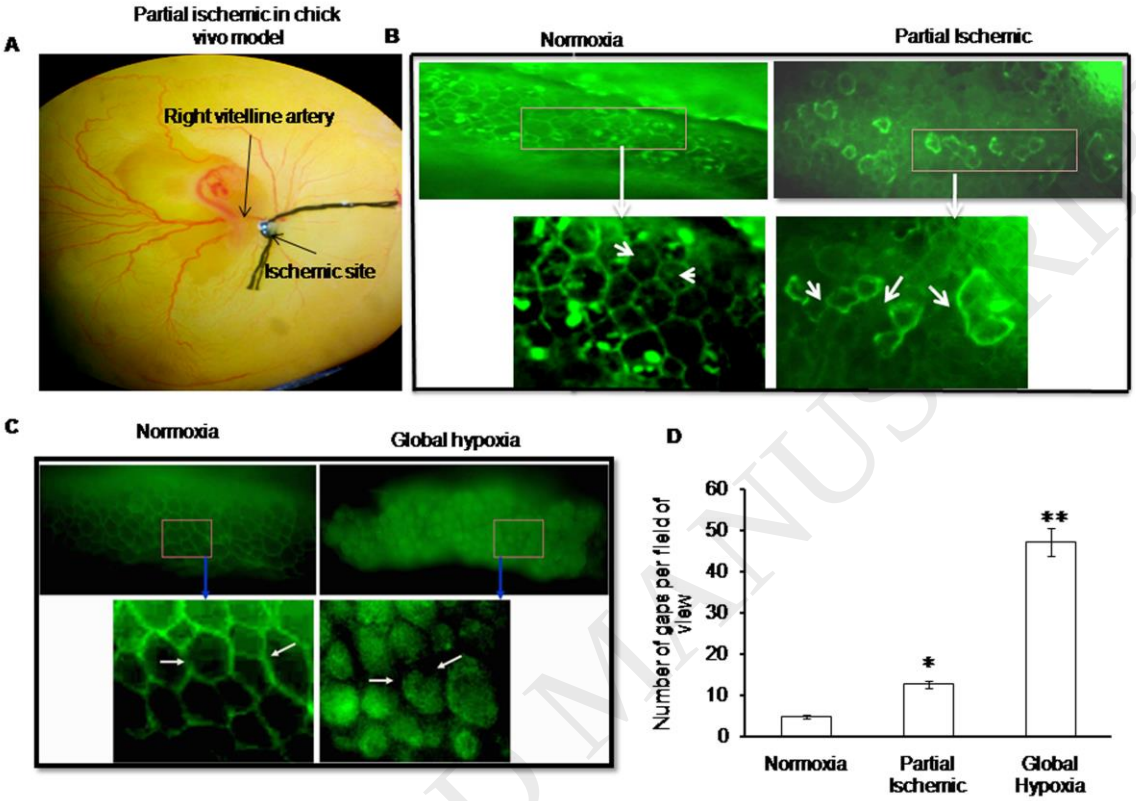


Figure.1

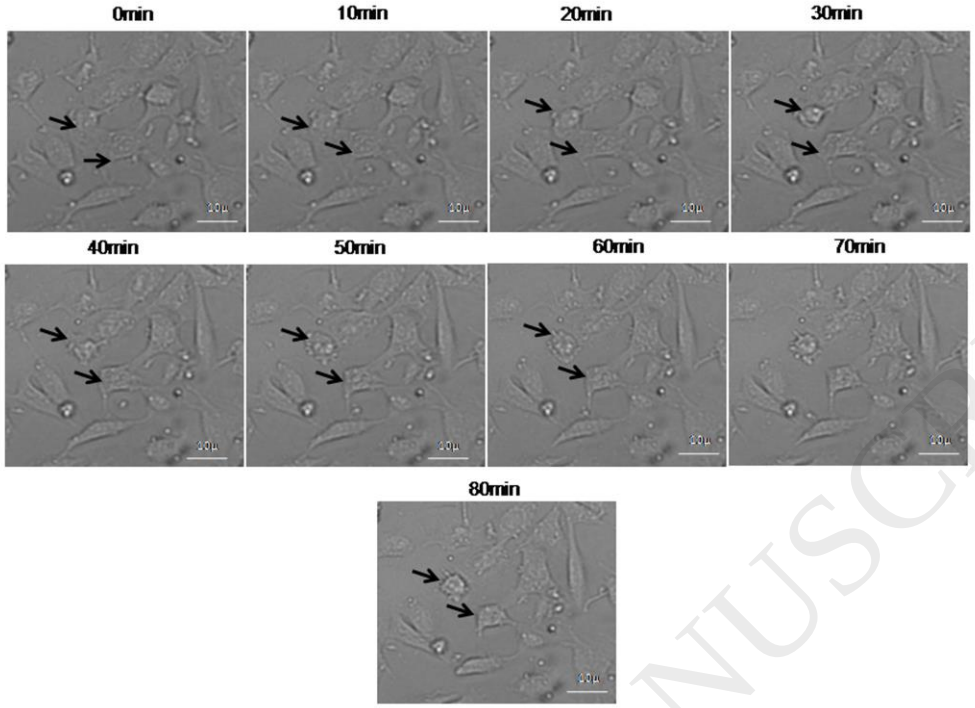


Figure.2

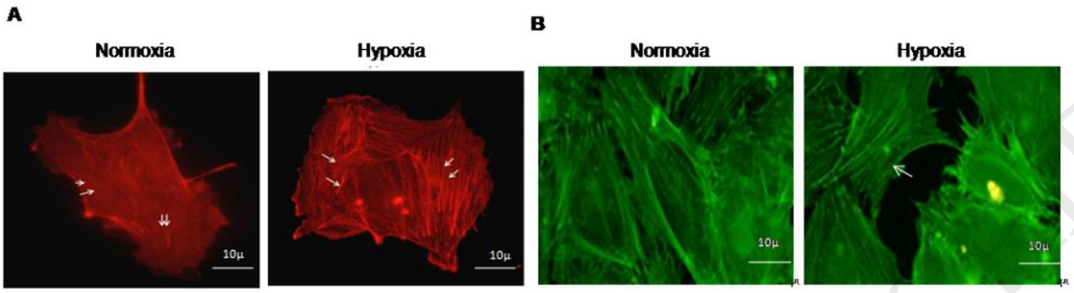


Figure. 3

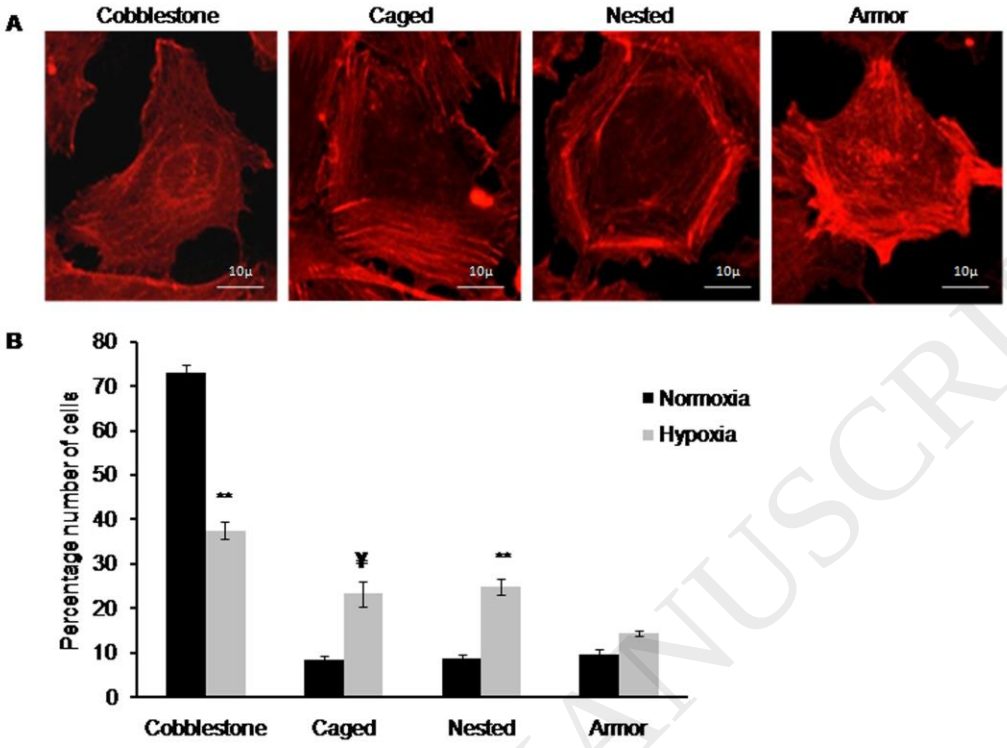


Figure. 4A,B

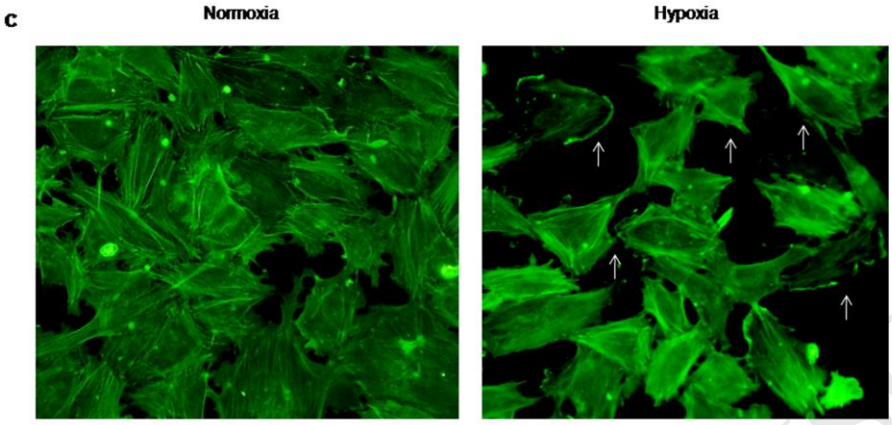


Figure. 4C

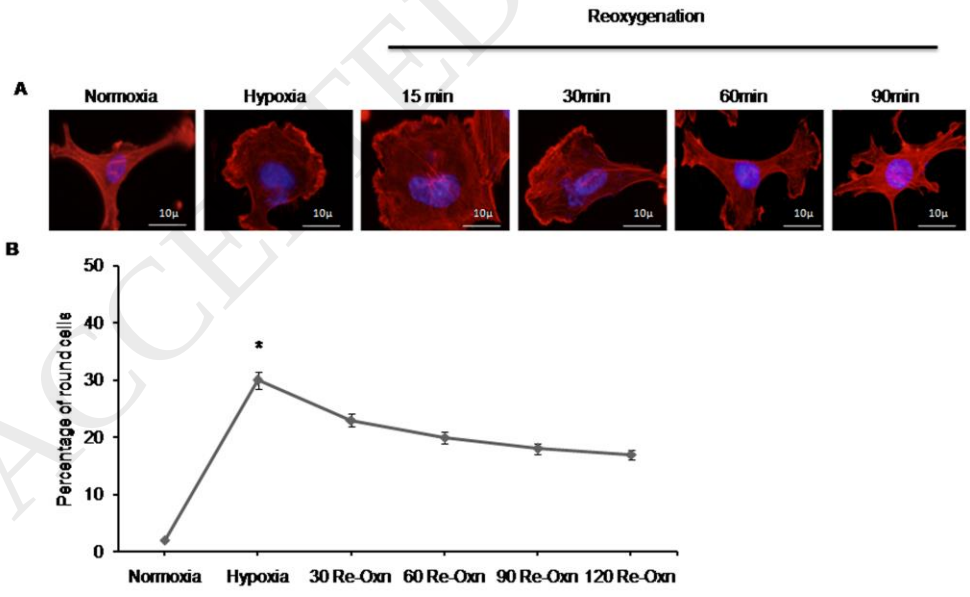
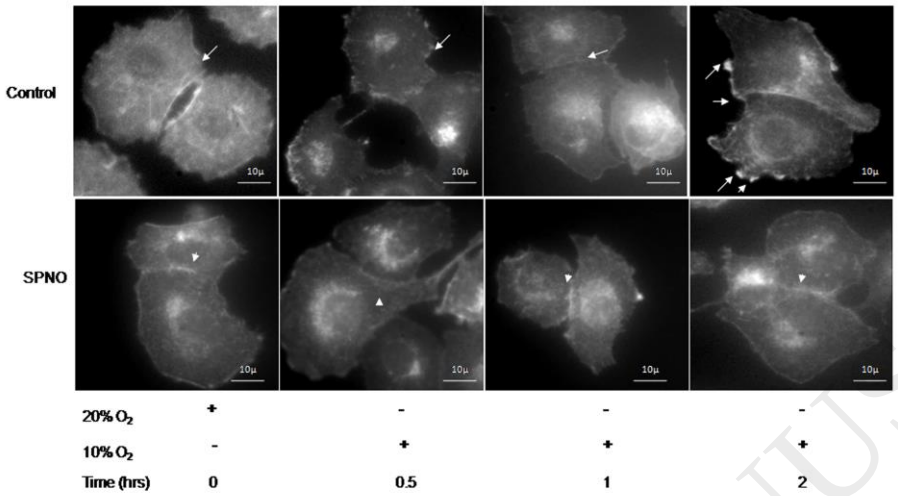


Figure. 5



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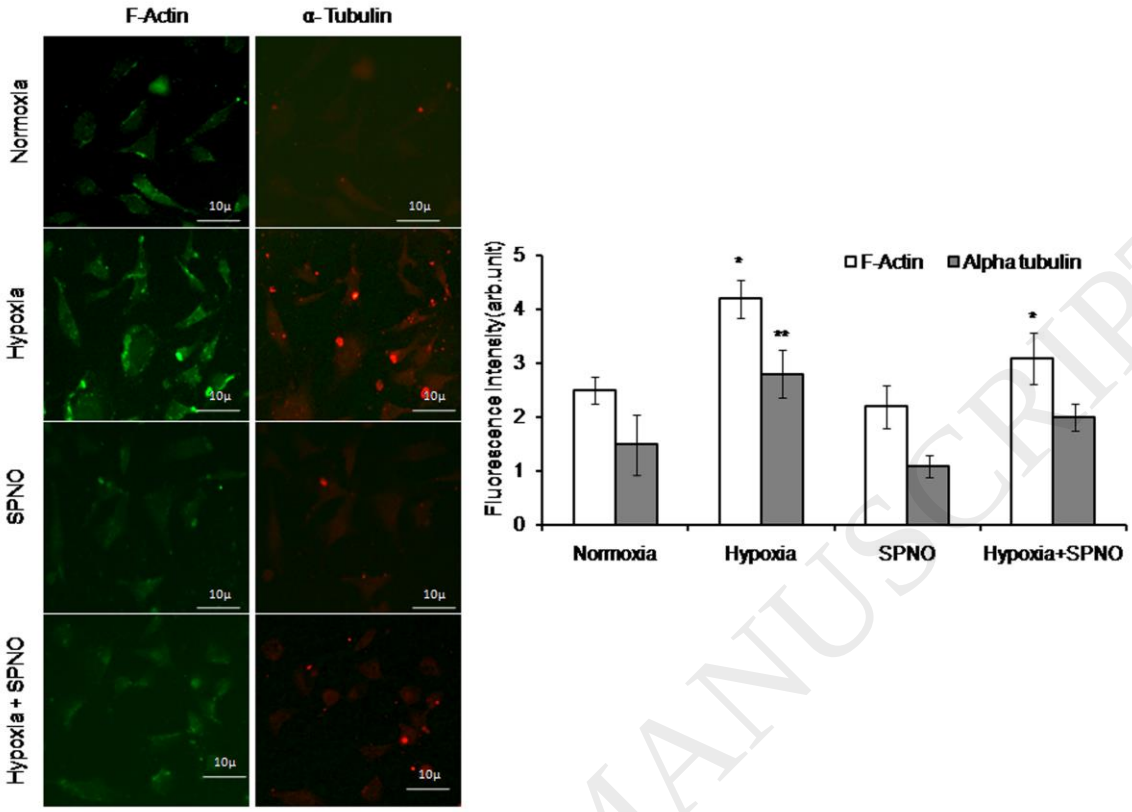

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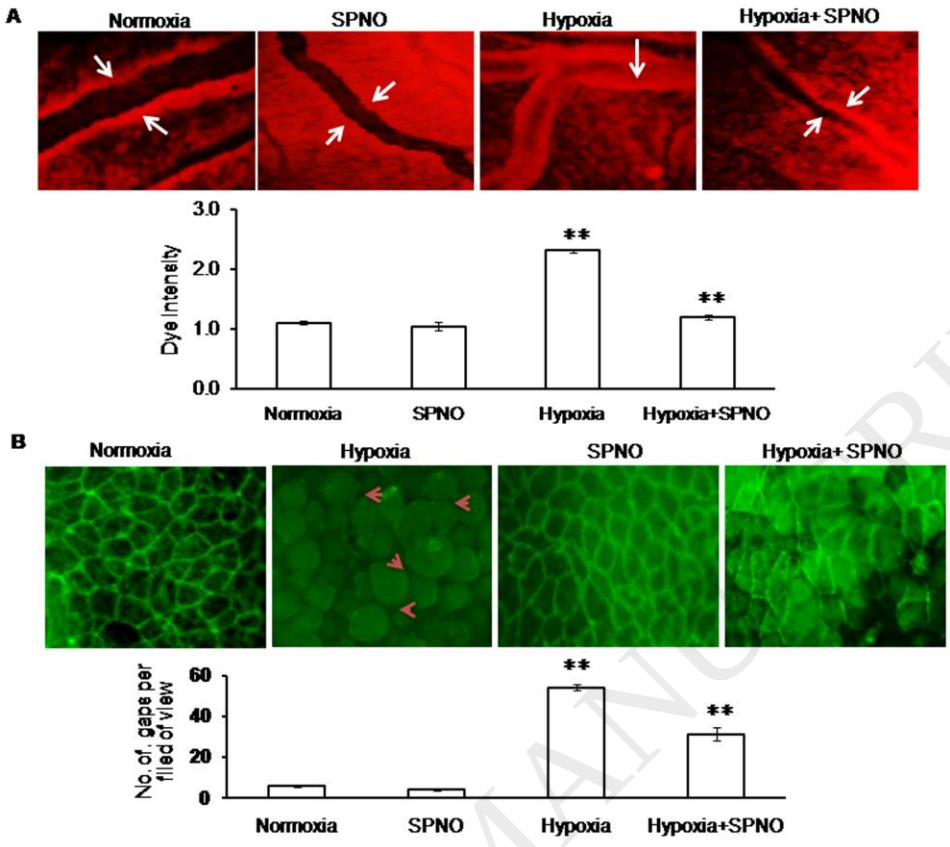

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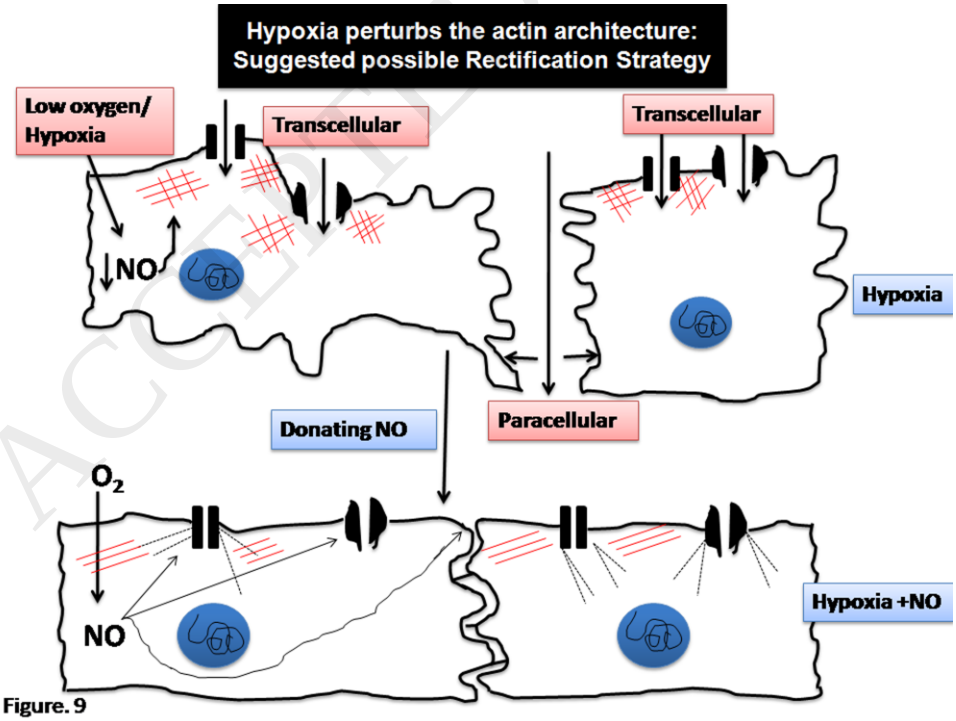

Figure. 9