Umbilical Mesenchymal Stromal Cells Provide Intestinal Protection through Nitric Oxide Dependent Pathways

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KEY WORDS

umbilical mesenchymal stromal cells, nitric oxide, endothelial nitric oxide synthase, ischemia reperfusion injury,

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ABSTRACT

Background: Umbilical-derived mesenchymal stromal cells (USC) have shown promise in the protection of ischemic organs. We hypothesized that USCs would improve mesenteric perfusion, preserve intestinal histological architecture, and limit inflammation by nitric oxide dependent mechanisms following intestinal ischemia/reperfusion injury (IR).

Methods: Adult wild type C57BL/6J (WT) and endothelial nitric oxide synthase knock out (eNOS KO) mice were used: 1) WT IR+Vehicle, 2) WT IR+USC, 3) eNOS KO IR+Vehicle and 4) eNOS KO IR+USC. Mice were anesthetized and a midline laparotomy was performed. The superior mesenteric artery was clamped with a non-occluding clamp for 60-minutes. Following IR, mice were treated with an injection of 250µL phosphate buffered saline (PBS) or $2x10^6$ USCs suspended in 250µL PBS. Mesenteric perfusion images were acquired using laser Doppler imaging. Perfusion was analyzed as a percentage of baseline. At 24 hours, mice were euthanized and intestines were harvested. Intestines were evaluated for injury and data were analyzed using the Mann-Whitney or Kruskal-Wallis tests.

Results: Intestinal mesenteric perfusion was significantly improved in WT mice treated with USC therapy compared to eNOS KOs. Intestinal histological architecture was preserved with USC therapy in WT mice. However, in eNOS KO mice, this benefit was abolished. Lastly, the presence of several cytokines and growth factors were significantly improved in WT mice compared to eNOS KO mice treated with USCs.

Conclusion: The benefits of USC-mediated therapy following intestinal IR injury likely occur via nitric oxide dependent pathways. Further studies are required to define the molecular mechanisms by which USCs activate endothelial nitric oxide synthase to bring about their protective effects.

INTRODUCTION

Acute mesenteric ischemia (AMI) continues to be a devastating intra-abdominal emergency with mortality as high as 60-80% and accounts for about 0.1% of all hospital admissions (1, 2). It is caused by either 1) a sudden acute arterial occlusion, 2) a venous occlusion or 3) a sudden drop in circulating pressure. During hypoperfusion, this insufficient blood flow within the mesenteric circulation is unable to meet intestinal metabolic demands, and often, this leads to mesenteric infarction and intestinal necrosis. Patients who remain

untreated can quickly decompensate and progress to shock, multi-system organ failure and death (3). The most critical factor that continues to impact outcomes in patients is the time to diagnosis and intervention.

If patients survive these ischemic episodes, they are often faced with prolonged hospitalization and long term parenteral nutrition needs (4). Additionally, reported overall survival at one, three and five years following surgery for AMI has been found to be 26%, 23%, and 21% respectively (5). Early diagnosis and aggressive therapy may significantly reduce the morbidity and mortality of this life-threatening disease. While clinical studies emphasize diagnostic and therapeutic algorithms to expedite treatment for the diagnosis of AMI, the disease prognosis remains dismal. To that end, noteworthy advancements in the medical treatment of intestinal ischemia within the last decade have been sparse and a novel therapeutic option is of the utmost importance.

Recent animal studies have demonstrated the ability to reverse ischemic injury and promote recovery of intestinal tissue with use of mesenchymal stromal cells (MSCs) following ischemia-reperfusion (IR) injury (3, 6-8). Umbilical-derived mesenchymal stromal cells (USCs) are pluripotent, immunomodulatory, and reduce inflammation (9). They are easily isolated from neonates and obtained from discarded tissue. Of all the types of mesenchymal stromal cells (adipose-derived, bone marrow-derived, etc.), they are the least senescent and have the lowest expression of MHC II, which may enhance cell survival during transplant (9).

Mesenchymal stromal cells originally were thought to allow for tissue repair through transdifferentiation or cell fusion. However, several studies have now demonstrated that functional benefits observed with use of MSCs following injury are most likely related to the secretion of bioactive mediators acting in a paracrine fashion on the affected tissue (10). Many of these factors include epidermal growth factor, insulin-like growth factor, transforming growth factor beta1, hepatocyte growth factor,interleukin6, interleukin10 and vascular endothelial growth factor (11, 12). While all of these paracrine mediators have been found to contribute to improved tissue repair and angiogenesis following ischemic injury, it is still unclear how they promote improved mesenteric perfusion following injury.

In this regard, nitric oxide (NO) could be an important downstream contributor to USC mediated improvements in mesenteric perfusion. Nitric oxide is known to be a potent vasodilator through relaxation of vascular smooth muscle cells. In the setting of intestinal IR injury, studies have found that endogenous nitric oxide production increases, most likely to promote improved perfusion to ischemic organs (13, 14). Within the

microvascular endothelial bed, nitric oxide is produced by endothelial nitric oxide synthase (eNOS). Therefore, it is possible that USCs may work through endothelial nitric oxide synthase pathways to bring about their protective effects. We hypothesized that: 1) USCs would improve mesenteric perfusion, preserve intestinal histological architecture, and limit intestinal inflammation following intestinal IR injury and 2) the benefits of USC therapy would be mediated through endothelial nitric oxide synthase-dependent pathways.

MATERIALS AND METHODS

Animals

Wild-type adult male mice (C57BL/6J, 8-12 weeks; Jackson Laboratory, Bar Harbor, ME) and endothelial nitric oxide synthase knock out (eNOS KO) mice on a C57BL/6J background (B6.129P2-Nos3tm1Unc/J, 8-12 weeks; Jackson Laboratory, Bar Harbor ME) were used for all animal experimentation. Animals underwent at least 48 hours of acclimation to the new environment prior to experimentation. Twelve hour light/dark cycle housing and normal chow and water were provided to all animals. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Indiana University.

Cell Culture

The human umbilical-derived mesenchymal stromal cells used for experimentation were purchased from ATCC (Manassas, VA). Cells were positive for CD29, CD44, CD73, CD90, CD105, and CD166 and negative for CD14, CD31, CD34 and CD45 (15). Cells were cultured in 225 cm^2 polystyrene culture flasks in Mesenchymal Stem Cell Basal medium with Mesenchymal Stem Cell Growth Kit – Low Serum (ATCC, Manassas, VA) at 37˚C in a humidified atmosphere of 5% CO_2 in air. Once cells reached 90% confluency they were lifted from the flask with TrypLE Express (Life Technologies) and passaged to expand primary cultures or used in experimentation. USCs were used between passages 4-9. A fluorescent automated cell counter was used to count cells (Luna™ Automated Cell Counter, Logos Biosystems Inc., Annandale, VA).

Ischemia/Reperfusion Model

For all experimental groups, mice were anesthetized using 3% isoflurane followed by maintenance at 1.5% isoflurane in oxygen. Temperature homeostasis was maintained intra-operatively through the use of an animal heating pad. All murine abdomens were prepped using hair removal lotion followed by sterile preparation with 70% ethanol and betadine. Prior to surgery all animals were subcutaneously injected with

one milliliter of 0.9% normal saline solution to account for intra-operative fluid loss. Peri-operative analgesia included 1 mg/kg buprenorphine and 5mg/kg carprofen administered by subcutaneous injection.

Using a previously described sterile technique, a midline laparotomy was performed and the intestines were eviscerated. Using an atraumatic microvascular clamp, the superior mesenteric artery was clamped to cause intestinal ischemia. The intestines were then returned to the abdominal cavity and the abdomen was closed temporarily for a total of 60 minutes to prevent evaporative losses. Following the ischemic period, the abdominal cavity was reopened and the atraumatic clamp was removed. Animals then received an intraperitoneal injection of 250 µL PBS as our vehicle control or $2x10^6$ umbilical-derived MSCs re-suspended in 250 µL of PBS. In a two-layer fashion, the abdominal fascia and skin were then closed with suture. Following surgery all animals were allowed to recover and awaken on a heating pad. Once they had fully recovered, they were returned to our laboratory animal resource center (3).

Perfusion Analysis

Animals were assigned to the perfusion protocol (N=10 per group) as follows: 1) WT IR + vehicle, 2) WT IR + USCs, 3) eNOS KO IR + vehicle, and 4) eNOS KO IR + USCs. Using a Laser Doppler Perfusion Imager (LDI; Moor Instruments, Wilmington, DE), mesenteric and intestinal perfusion was analyzed. Acquisition of images occurred at baseline, at initial clamping of the superior mesenteric artery and at 24 hours following reperfusion. Intestines were eviscerated and oriented in a standard fashion beneath the laser in order to eliminate bias. Using these images, a region of analysis was created around the eviscerated intestines to obtain a flux mean perfusion within this region. At each of the three time points a total of three images were acquired and averaged. The perfusion was then expressed as a percentage of baseline. Once perfusion images were obtained, animals were euthanized through overdose of isoflurane and cervical dislocation. Intestines were then harvested for further analysis (3).

Histological Injury Score

In all experimental groups, intestinal tissues were harvested following euthanasia (N=10 per group). The terminal ileums were fixed using 4% paraformaldehyde with subsequent dehydration in 70% ethanol. Intestines were paraffin-embedded, sectioned, and subsequently stained with hematoxylin and eosin. A histological scoring method of intestinal damage was used as previously described: 0, no damage; 1, subepithelial space at the villous tip; 2, loss of mucosal lining at the villous tip; 3, loss of less than half of the

villous structure; 4, loss of more than half of the villous structure; and 5, transmural necrosis (16, 17) . All histological sections were evaluated by two blinded authors (ARJ, NAD) and scores were averaged.

Intestinal Cytokine Analysis

Following euthanasia, all mouse intestinal tissue was harvested and snap frozen in liquid nitrogen and stored at -80ºC for later use. For protein analysis, intestines were thawed and homogenized in RIPA buffer (Sigma, St. Louis, MO) with phosphatase and protease inhibitors (1:100 dilution, Sigma, St. Louis, MO). A Bullet Blender tissue homogenizer (Next Advance, Averill Park, NY) was used for tissue homogenization. After homogenization, samples were centrifuged at 12,000 rpm to pellet extraneous tissue. Supernatants were then collected in fresh Eppendorf tubes and total protein concentration was quantified with the Bradford Assay using a spectrophotometer (VersaMax microplate reader; Molecular Devices, Sunnyvale, CA).

For measurement of murine intestinal inflammatory cytokine and growth factor production, a Bio-Plex 200 multiplex beaded assay system (Bio-Rad, Hercules, CA) was used. The multiplex assay was ran a total of three times (N=13-25 per group based on plate restrictions). Assays were performed at 1:25 dilution according to the manufacturer's instructions. Assayed cytokines and growth factors included interleukin 6 (IL-6), interleukin 9 (IL-9), interleukin 10 (IL-10), C-X-C ligand 10 (IP-10), granulocyte-colony stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and angiopoietin 2 (ANPT-2). In order to account for variations in individual plates and assays, cytokines were normalized to their respective vehicle controls for both WT and eNOS KO intestinal samples.

Statistical Analysis

GraphPad Prism 7 (GraphPad Software, La Jolla, CA) was used for all statistical analysis and figures. For power analysis we used a confidence level of 95% and a power of 80%. We estimated there would be a 50% difference between treatment and vehicle groups. Therefore, based on our analysis, a minimum of 8 animals would be needed per group. For the study we used a total of 10 animal in each group. To account for any outliers, prior to data analysis, raw data was assessed by robust regression and outlier removal (ROUT method). Groups were compared using Mann-Whitney and Kruskal-Wallis tests for nonparametric variables. P-values less than 0.05 were considered statistically significant.

RESULTS

Mesenteric Perfusion is improved with USC Therapy following IR through eNOS Dependent Pathways

Intestinal mesenteric perfusion was compared at 24 hours following IR injury in all treatment groups (Figure 1A). One animal in each group was removed after performing analysis of outliers (ROUT method). This left nine animals per group for perfusion analysis. Treatment with USCs significantly improved mesenteric perfusion in the wild-type animals compared to vehicle treatment alone (WT Vehicle=32.6±7.7, WT USCs=68.2±5.0, p=0.02). While there was significant improvement in perfusion in WT animals with USC therapy, this was not observed in the eNOS KO animals (eNOS KO Vehicle=32.7±6.1, eNOS KO USCs=37.9±11.8, p=>0.9; Figure 1B).

Histological Architecture is preserved with USC Therapy following IR through eNOS Dependent Pathways

Intestinal architecture was compared at 24 hours following IR injury (N=10/group, Figure 2A). In wild-type animals, USC therapy significantly improved the preservation of intestinal architecture compared vehicle alone (WT Vehicle=3.4±0.3, WT USCs=0.6±0.2, p=<0.01). Additionally, while there was preservation of the intestinal architecture in WT animals following USC therapy, this was not observed in eNOS KO animals (eNOS KO Vehicle=4.0±0.2, eNOS KO USCs=3.0±0.2, p=0.1; Figure 2B).

USCs Increase Cytokine Production following IR Injury through eNOS Dependent Pathways

Intestinal inflammatory cytokine production following IR injury was significantly impacted by USC therapy and these effects were found to be mediated through eNOS dependent pathways (Figures 3). IL-9 production after USC therapy was significantly decreased in eNOS KO animals compared to WT animals (WT = 1.5±0.2, eNOS KO = 0.9±0.1; p=<0.01). The production of IL-10, a critical anti-inflammatory cytokine, was significantly decreased in eNOS KO animals compared to WT animals after USC therapy (WT = 1.6±0.2, eNOS KO = 0.6±0.1; p=<0.01). Lastly, the production of IP-10, a known cellular chemoattractant, was significantly decreased with USC therapy in eNOS KO animals compared to WT (WT = 1.0±0.1, eNOS KO = 0.4±0.0; p=<0.01). IL-6 levels following USC therapy was not significantly different between WT and eNOS KO animals (WT = 0.7±0.3, eNOS KO = 0.2±0.1; p=0.6).

USCs Promote Growth Factor Production following IR Injury through eNOS Dependent Pathways

Intestinal production of multiple growth factors following USC therapy was also found to be mediated through eNOS dependent pathways (Figure 4). G-CSF was significantly decreased with USC therapy in eNOS KO animals (WT = 0.4±0.1, eNOS KO = 0.1±0.0; p=0.02), while HGF was significantly increased in eNOS KO

animals compared to WT animals (WT = 0.4±0.1, eNOS KO = 0.6±0.1; p=0.02). In addition, intestinal VEGF and ANGPT-2 levels were significantly decreased in eNOS KO animals compared to WT (VEGF: WT = 1.2±0.1, eNOS KO = 0.7±0.1; p=<0.01, ANGPT-2: WT = 1.1±0.2, eNOS KO = 0.5±0.1; p=<0.01).

DISCUSSION

Acute mesenteric ischemia continues to be a devastating intra-abdominal emergency with high morbidity and mortality and limited ability to salvage ischemic bowel. While current management focuses on resuscitation, embolectomy, revascularization and resection; there is no available treatment modality aimed at recovery of the already ischemic intestine. Therefore, novel therapies are being explored. To this end, we have observed that umbilical-derived mesenchymal stromal cell therapy for intestinal ischemia may allow for recovery of the damaged tissues and allow for intestinal preservation. Additionally, these protective effects may be mediated, in part, by endothelial nitric oxide synthase dependent pathways.

This study demonstrated that intraperitoneal USC therapy following intestinal IR injury improved mesenteric perfusion, prevented intestinal mucosal damage, and increased the production of certain inflammatory mediators associated with injury. Our findings corroborate similar findings in other studies of MSC therapy for intestinal ischemia that observed reduced intestinal barrier disruption (3, 18), improved mesenteric perfusion (3, 6), suppression of pro-inflammatory cytokine production, and increased expression of anti-inflammatory cytokines (3, 6, 8).

While all of these studies have demonstrated intestinal recovery after IR with use of MSCs, there have been limited studies specifically examining the role of nitric oxide as a downstream mediator in relation to intestinal protection by USCs. Herein, we found that improvements in mesenteric perfusion and preservation of intestinal architecture were lost when eNOS was genetically ablated. Our findings suggest that nitric oxide production plays a key role in the downstream benefits of MSC therapy. Similarly, in a swine model of myocardial IR, Song et al. demonstrated that the benefits of MSC therapy were in part mediated by activation of eNOS, and observed that benefits of MSCs were abated with use of a NOS inhibitor (19). Additionally, other studies have confirmed that nitric oxide concentrations increase with ischemia and play a protective role in limiting tissue damage (13, 14).

Nitric oxide is a gasotransmitter known to play a key role in angiogenesis and vasorelaxation. Within the endothelium, eNOS uses L-arginine as a precursor to form nitric oxide (20). NO can then diffuse into

neighboring smooth muscle cells to activate soluble guanylate cyclase which then converts guanosine triphosphate into cyclic guanosine monophosphate (cGMP). cGMP then activates Protein Kinase G which has a number of downstream targets that work to cause smooth muscle cell relaxation (20).

By using competitive eNOS inhibitors such as NG-monomethyl-L-arginine (LNMMA) and NG-nitro-L-arginine-methyl ester (L-NAME), previous studies have highlighted the importance of eNOS in the control of vasodilation and angiogenesis (21). In a model of hind-limb ischemia, eNOS deficient mice were found to not only develop a severe form of critical limb ischemia, but also to exhibit impaired wound healing and defective angiogenesis (22, 23).The role that eNOS plays on angiogenesis is likely through downstream mediators of VEGF, as previous work has demonstrated impaired angiogenesis in eNOS KO mice following hind limb ischemia even when VEGF was administered exogenously (23). Other studies have also found that the production of VEGF enhances the expression of eNOS in endothelial cells possibly through post-transcriptional effects on eNOS mRNA stability (24). This up-regulation in expression may be important in the process of VEGF-induced angiogenesis. In this current study, we observed a significant decrease in the intestinal angiogenic factors VEGF and ANGPT-2 following USC therapy in eNOS KO animals compared to wildtypes. These findings may suggest a critical relationship between stromal cell therapy, eNOS production, and angiogenesis in that USCs may not be able to effectively facilitate angiogenesis in the absence of eNOS.

Multiple other cytokines were also measured following intestinal IR injury. We observed a significant decrease in the production of IL-9, IL-10 and IP-10 in eNOS KO animals compared to WT animals following USC therapy. IL-9 is known to regulate activated T-cell proliferation and immunoglobulin production (25). Additionally, it is known to promote mast cell proliferation and cytokine secretion. While it was significantly increased following USC therapy in the WT group, this was not observed in the eNOS KO animals suggesting that the role of IL-9 in inflammatory disease may in part be regulated by eNOS dependent mechanisms. IL-10, a known anti-inflammatory cytokine was similarly increased with USC therapy in WT animals compared to eNOS KO animals. This anti-inflammatory cytokine has been found to be upregulated in other studies of MSC therapy following ischemic injury (26) and likely plays a protective role by limiting intestinal inflammation (27). Similarly, significant elevations in IP-10, a known chemoattractant, were observed in WT animals compared to eNOS KO animals following therapy. The significant decrease in these cytokines following USC therapy in eNOS KO mice suggests a dependency on eNOS for their production.

Lastly, HGF production was elevated following USC therapy in eNOS KO animals compared to wildtypes. HGF is known to stimulate chemotactic migration of MSCs and it is thought that it may play an important role in MSC recruitment to sites of tissue regeneration (28). Consequently, eNOS may play a role in suppressing this pathway under normal situations, possibly as a way to provide a check and balance pathway in tissue regeneration. Overall, the differences in production of growth factors and cytokines between wildtype and eNOS KO reinforce the idea that multiple complex pathways are used by MSCs to enhance recovery and allow for tissue repair following injury.

LIMITATIONS

This study has several limitations that may affect the impact of the results. First, for this study, human umbilical-derived mesenchymal stromal cells were utilized as a preclinical assessment in a mouse model of intestinal IR injury. In immunocompetent hosts, cross species transplantation is usually not possible and these beneficial effects are usually not observed. However, it is known that MSCs have the unique ability to suppress T-lymphocyte proliferation and thus allow for xenotransplantation (7, 29).

Additionally, the superior mesenteric artery occlusion model of intestinal IR injury is not the most representative of what is commonly seen in the clinical setting. While complete small bowel ischemia is certainly devastating and quite possible through an SMA thrombus or embolus, intestinal ischemia more commonly only affects a portion of the small bowel. Nevertheless, this model allows for the most severe form of intestinal injury to be used for examination of the effectiveness of various therapeutic strategies.

Lastly, despite normalizing for total protein concentration in intestinal tissue a wide variety of cytokine levels were observed between samples. While we used similar amounts of intestine from similar areas of the gastrointestinal tract, it is possible that there could be certain segments of ischemic bowel that have higher or lower levels of cytokine production and are therefore not completely equivalent in this investigation.

CONCLUSION

In conclusion, direct intraperitoneal application of USCs in the setting of acute mesenteric ischemia is an innovative approach to a very devastating and morbid disease. Herein, we demonstrated that USCs improve mesenteric perfusion, preserve intestinal histological architecture and moderate intestinal inflammatory cytokine and chemokine production. Additionally, these beneficial effects appear to be mediated through endothelial nitric oxide synthase pathways. While these benefits have been observed and it has

become clear that eNOS plays a significant role in tissue recovery following injury, we still do not know all of the downstream effects of these pluripotent immunomodulatory cells. Therefore, additional studies must be done to further investigate and delineate these mechanisms prior to clinical application.

FIGURE LEGENDS

Figure 1. Mesenteric perfusion following intestinal IR and USC therapy. A. Representative images from Laser Doppler Imager at 1) baseline, 2) SMA occlusion, and 3) 24 hours after ischemic injury in each respective treatment group (WT IR + Vehicle, WT IR + USC, eNOS KO + Vehicle and eNOS KO + USC). B. USC therapy significantly improved mesenteric perfusion in WT animals compared to eNOS KO mice at 24 hours reperfusion (*p=<0.05 vs. WT IR + USCs).

Figure 2. Histological examination of small intestine following intestinal IR injury and USC therapy. A. Representative histology slides of each treatment group ((WT IR + Vehicle, WT IR + USC, eNOS KO + Vehicle and eNOS KO + USC; hematoxylin and eosin stain x20). B. Histological scoring of intestinal specimens: 0, no damage; 1, sub-epithelial space at the villous tip; 2, loss of mucosal lining of the villous tip; 3, loss of less than half of the villous structure; 4, loss of more than half of the villous structure; and 5, transmural necrosis. USC therapy significantly improved histological mucosal injury grade in WT animals compared to eNOS KO mice at 24 hours reperfusion (*p=<0.05 vs. WT IR + USCs).

Figure 3. Intestinal cytokine analysis following IR injury and USC therapy at 24 hours reperfusion. All cytokine production is presented as a ratio of USC therapy/PBS Vehicle therapy in both the WT and eNOS KO animals. Following intestinal IR injury mouse intestines were homogenized and assessed for murine expression of IL-6, IL-9, IL-10, and IP-10 (*p=<0.05 vs. WT).

Figure 4. Intestinal growth factor production following IR injury and USC therapy at 24 hours reperfusion. All growth factor production is presented as a ratio of USC therapy/PBS Vehicle therapy in both WT and eNOS KO animal. Following intestinal IR injury mouse intestines were homogenized and assessed for murine expression of HGF, G-CSF, VEGF, and ANPT-2 (*p=<0.05 vs. WT).

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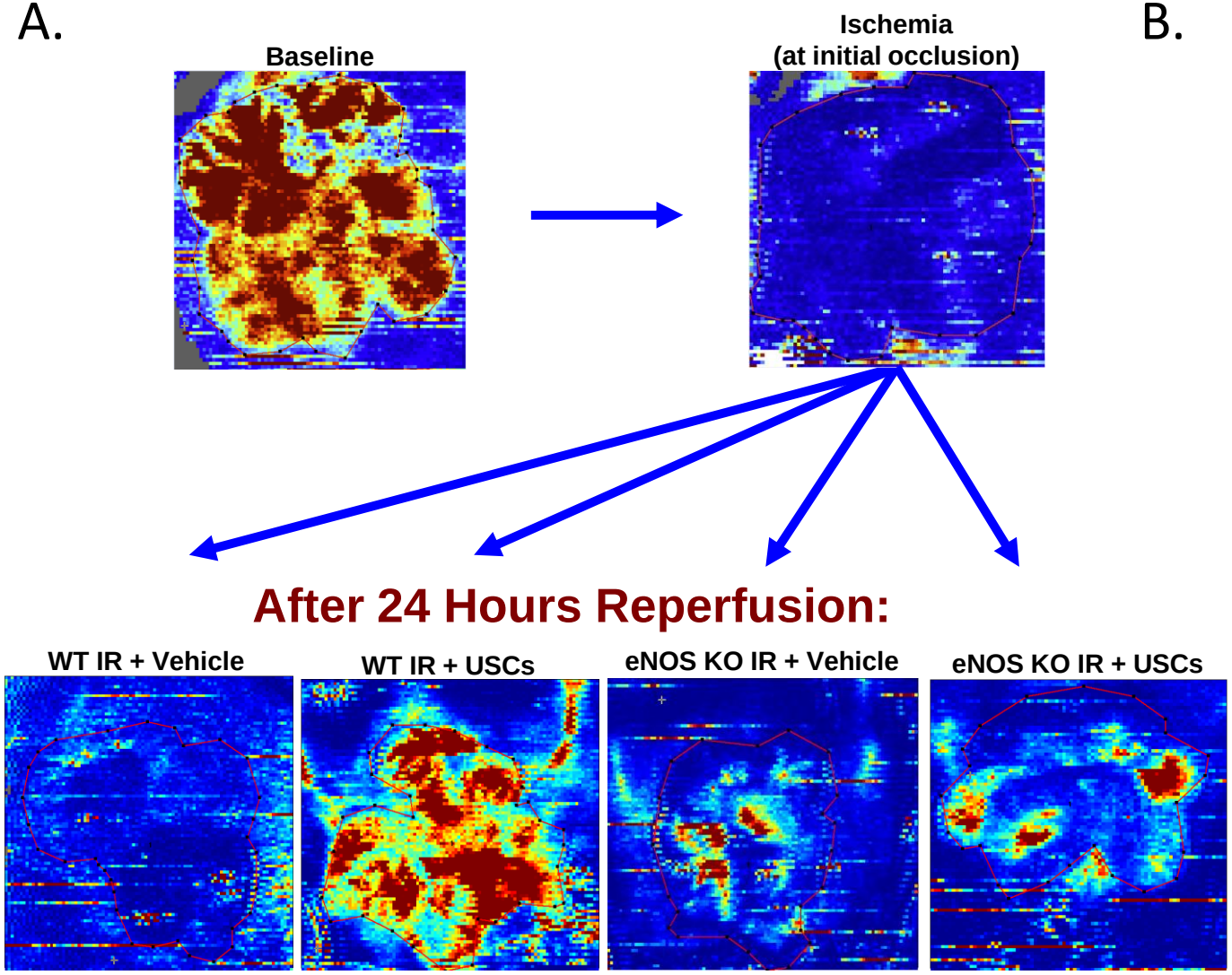
Figure 1.

A.


Baseline
Ischemia
(at initial occlusion)
After 24 Hours Reperfusion:
WT IR + Vehicle
WT IR + USCs
eNOS KO IR + Vehicle
eNOS KO IR + USCs

B.

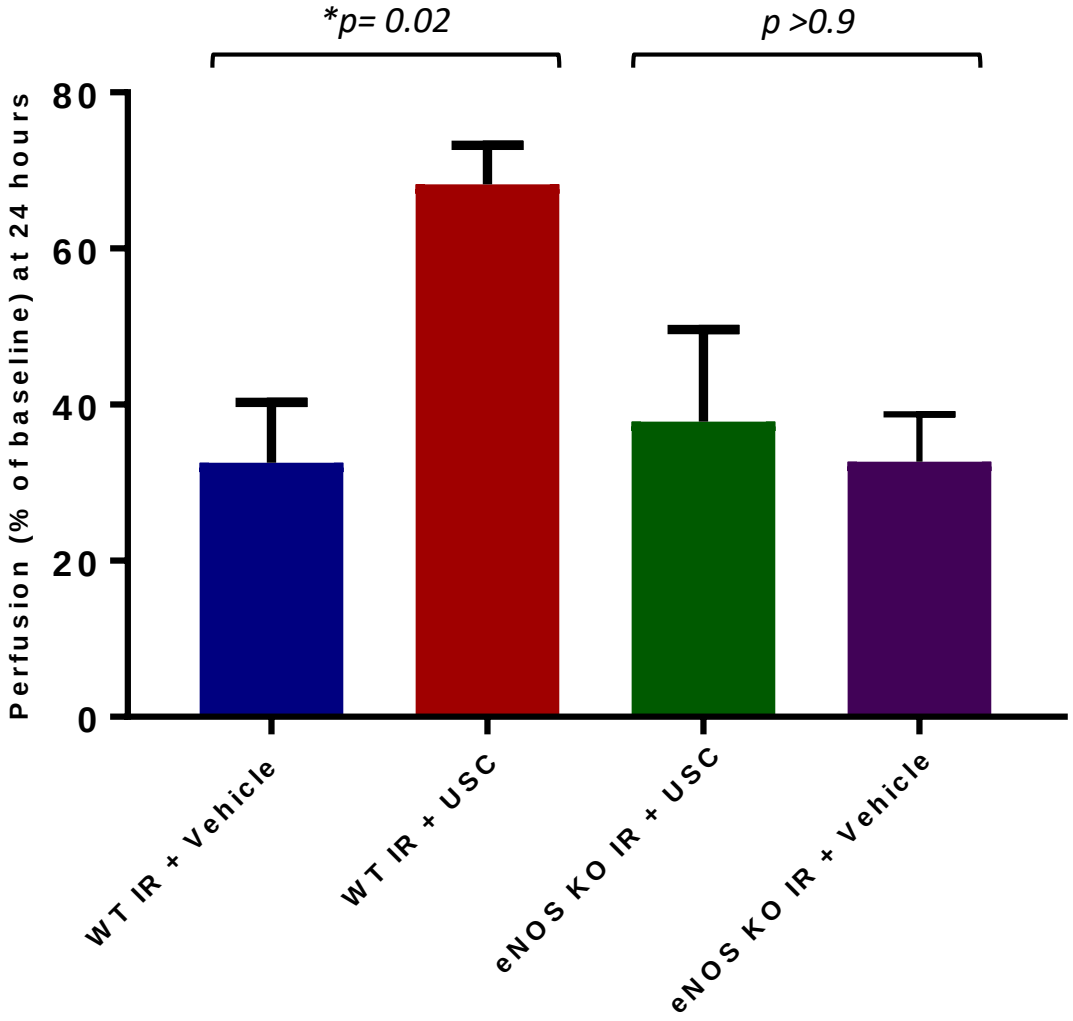
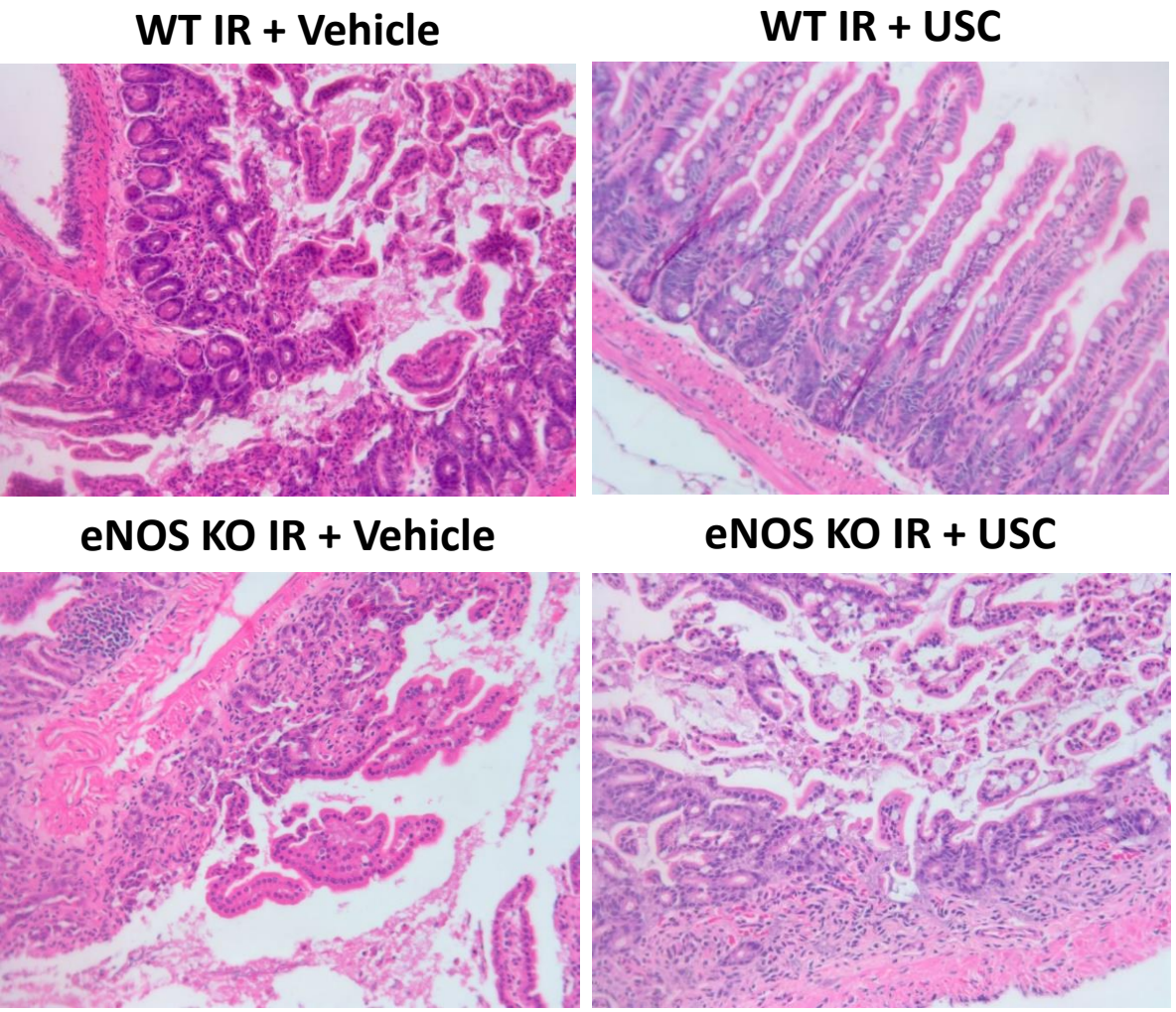

*p= 0.02
p >0.9
Perfusion (% of baseline) at 24 hours
80
60
40
20
0
WT IR + Vehicle
WT IR + USC
eNOS KO IR + USC
eNOS KO IR + Vehicle

Figure 2.

A.


WT IR + Vehicle
WT IR + USC
eNOS KO IR + Vehicle
eNOS KO IR + USC

B.

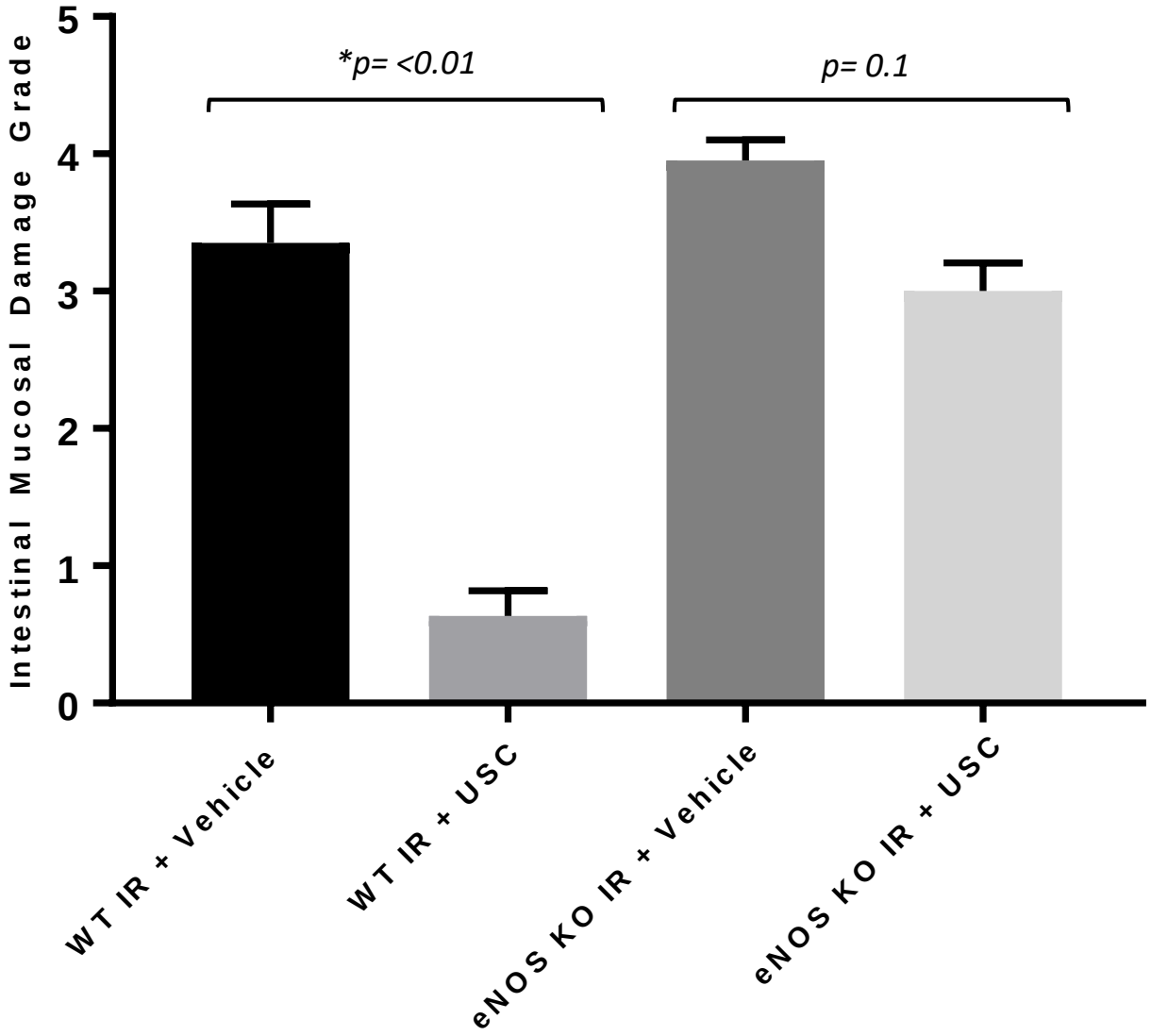
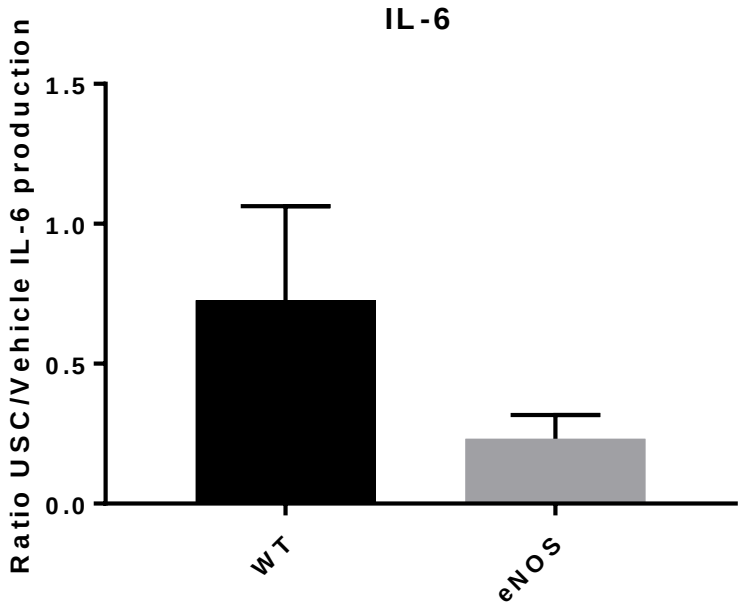
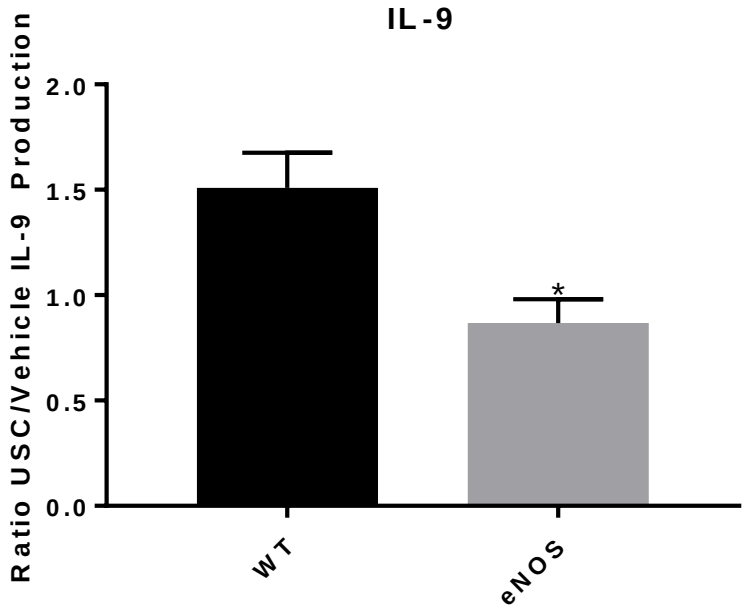
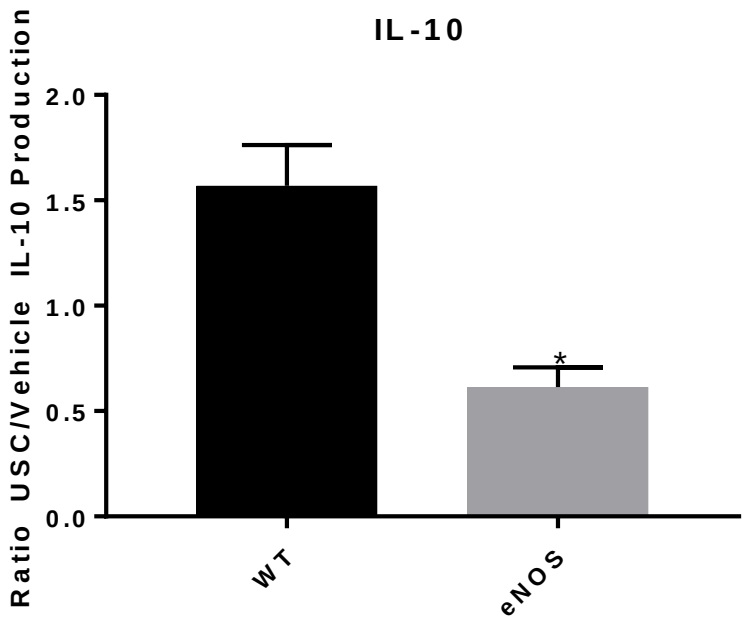

Intestinal Mucosal Damage Grade
0
1
2
3
4
5
*p= <0.01
p= 0.1
WT IR + Vehicle
WT IR + USC
eNOS KO IR + Vehicle
eNOS KO IR + USC

Figure 3.


IL-6
Ratio USC/Vehicle IL-6 production
1.5
1.0
0.5
0.0
WT
eNOS


IL-9
Ratio USC/Vehicle IL-9 Production
2.0
1.5
1.0
0.5
0.0
*
WT
eNOS


IL-10
Ratio USC/Vehicle IL-10 Production
2.0
1.5
1.0
0.5
0.0
*
WT
eNOS

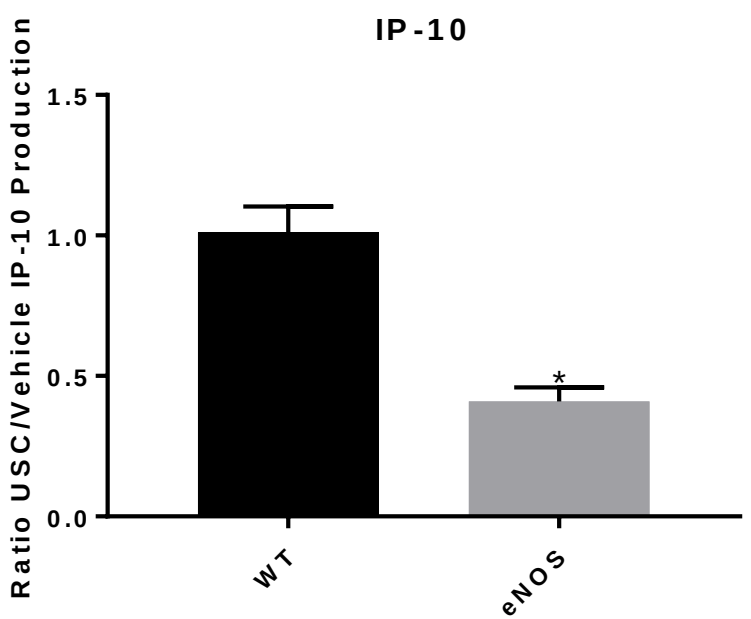
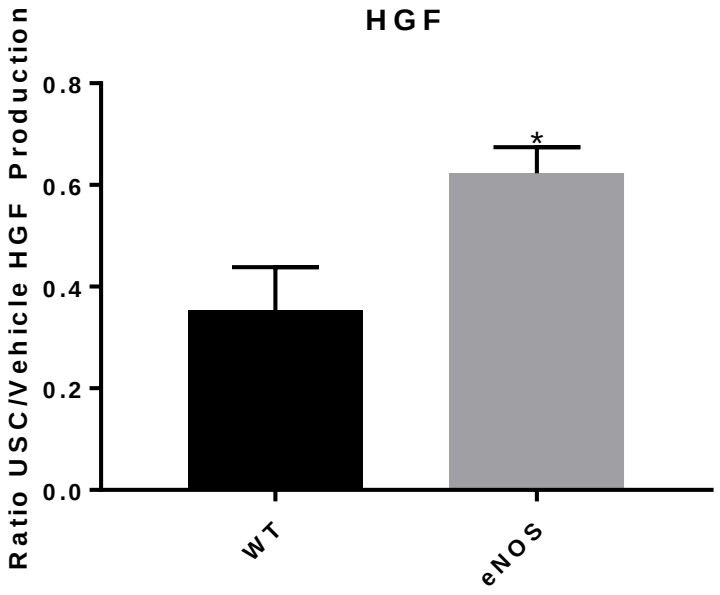
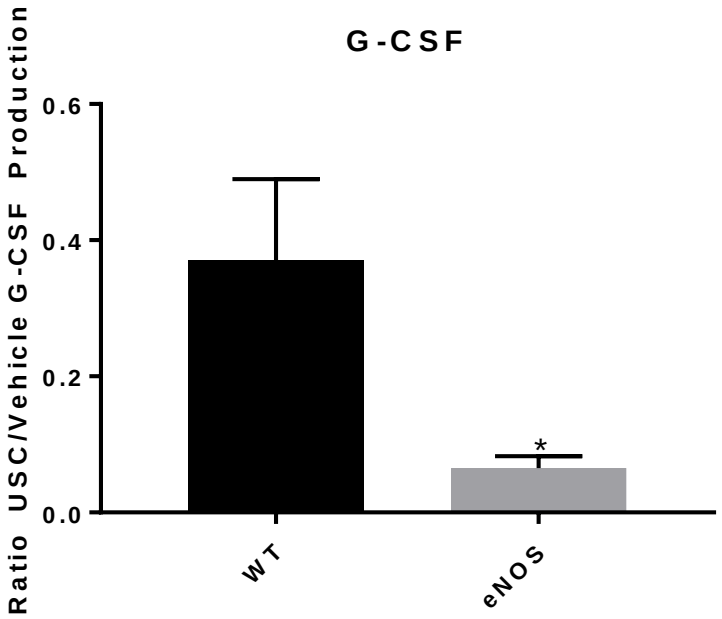
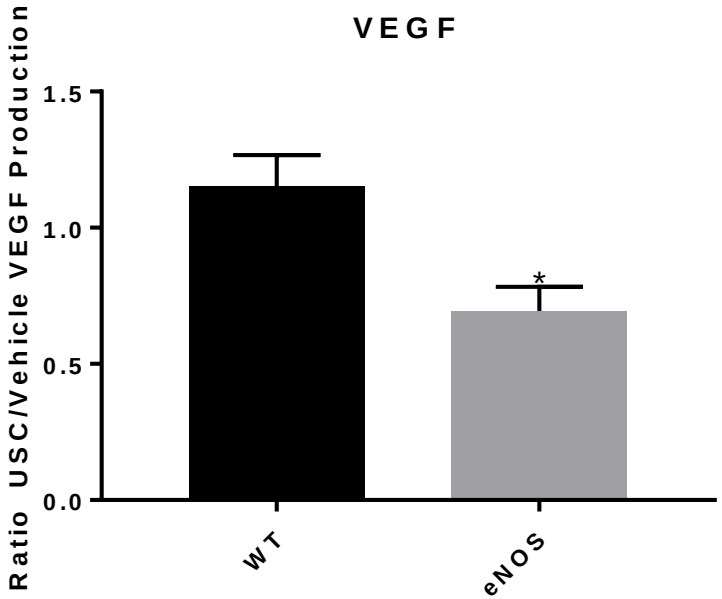
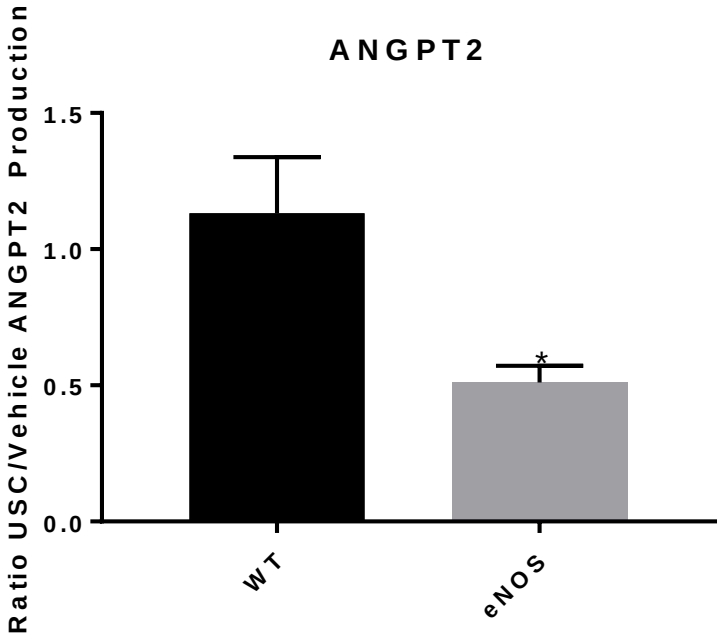

IP-10
Ratio USC/Vehicle IP-10 Production
1.5
1.0
0.5
0.0
*
WT
eNOS

Figure 4.


HGF
Ratio USC/Vehicle HGF Production
0.8
0.6
0.4
0.2
0.0
*
WT
eNOS


G-CSF
Ratio USC/Vehicle G-CSF Production
0.6
0.4
0.2
0.0
*
WT
eNOS


VEGF
Ratio USC/Vehicle VEGF Production
1.5
1.0
0.5
0.0
*
WT
eNOS


ANGPT2
Ratio USC/Vehicle ANGPT2 Production
1.5
1.0
0.5
0.0
*
WT
eNOS