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Interleukin-6 Therapy Improves Intestinal Recovery Following Ischemia

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Abstract

Purpose—Interleukin-6 (IL6) has both pro-and anti-inflammatory pathways but its effects on intestinal recovery following ischemia are unknown. We hypothesized that administration of IL6 following intestinal ischemia would improve mesenteric perfusion and mucosal injury.

Methods—Adult male C57Bl6J mice were anesthetized and a laparotomy performed. Baseline intestinal perfusion was assessed by laser Doppler imaging. Intestinal ischemia was induced for 60 minutes by temporarily occluding the superior mesenteric artery. After ischemia, treatments were administered intraperitoneally before closure (Vehicle: 250 μL phosphate-buffered-saline (PBS), IL6 low dose (20ng), IL6 medium dose (200ng), or IL6 high dose (2ug)). Animals were allowed to recover for 24 hours, were re-anesthetized and their mesenteric perfusion reassessed. Perfusion was expressed as percentage of baseline. Animals were then sacrificed, and intestines explanted for histological analysis. Separate frozen samples were homogenized and analyzed by ELISA for Vascular Endothelial Growth Factor (VEGF) and Interferon Gamma-Induced Protein 10 (IP10).

Results—IL6 increased mesenteric perfusion in low dose groups only, while it improved post-ischemic mucosal injury scores in both low and medium dose groups. No differences in perfusion or histology were seen when high dose IL6 was utilized. Intestinal VEGF was higher in the low dose IL6 group compared to vehicle, while IP-10 levels were lower in low and medium dose groups compared to vehicle. No differences were noted compared to vehicle in intestinal VEGF and IP-10 with high dose IL6 therapy.

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Conclusion—Lower doses of IL6 may serve as effective therapy to decrease intestinal injury after ischemia. Further studies are needed to elucidate the downstream mechanisms prior to widespread clinical use.

Keywords

Intestinal ischemia; IL-6; reperfusion injury; IP-10; VEGF

INTRODUCTION

Acute intestinal ischemia is a devastating disease with a wide variety of etiologies affecting all population groups. The annual incidence is 0.09 to 0.2 % per year [1]. Despite its relatively low incidence, the consequences of intestinal ischemia are severe. Sequelae can include extensive intestinal tissue damage which may require prompt surgical intervention to remove necrotic bowel. If an extensive amount of intestine needs to be removed, patients can be left with a less than optimal amount of intestine to absorb nutrition. This may require long term parental nutrition or intestinal transplantation. Left untreated, the mortality rate can reach fifty percent [2].

The development of new medical treatment options for intestinal ischemia have been sparse. Therefore, novel treatments are desperately needed. Chronic Interleukin-6 (IL6) administration has been shown to promote intestinal hyperplasia. One study demonstrated a 40% increase in small bowel mass and villus height associated with IL6 [3]. In another study, exogenous human IL-6mutein (IL-6m), a recombinant variant of human IL-6, was given to rats subcutaneously before small intestinal transplantation. Survival rates increased in the group treated with IL-6m compared to the control group by increasing perfusion in the intestinal tissue, decreasing polymorphonuclear cell migration, and reducing pro-inflammatory cytokine up-regulation [4]. Therefore, the use of IL6 for the treatment of acute intestinal ischemia may be beneficial to patient outcomes.

IL6 is a pleotropic enzyme of 184 amino acids that was first recognized as a soluble factor necessary to induce B-cell growth and differentiation [5]. It is known for a wide range of biological activities, with both anti-inflammatory and pro-inflammatory effects. Two different signaling processes mediate these effects. The classic, or cis-signaling, mediates the anti-inflammatory response whereas the pro-inflammatory effects are mediated by the alternative, or trans-signaling, mechanism (Figure 1). Both pathways require one molecule of IL-6, one IL-6 receptor (either membrane bound or soluble) and two molecules of gp130. [6].

The cellular effects of these two pathways have been studied in different tissues following ischemia. Knocking down the production of IL6 from adipose derived stem cells decreased skin flap recovery in mice through the classic signaling pathway [7]. Adverse effects were also seen in cardiac muscle remodeling after blocking the IL6 receptor following myocardial ischemia [8]. Conversely, beneficial effects surrounding cardiomyocyte apoptosis were seen following the administration of IL6/sIL6R complex [9]. Lastly, studies utilizing a trans-isomer fusion protein of IL6 and sIL6R demonstrated improved cellular regeneration following injury [10], thereby suggesting that the trans-signaling process may be also

involved in the beneficial effects of this cytokine. These studies seem to suggest a balance between the two pathways that could be exploited for therapeutic use.

The interaction of IL6 with its receptor induces a downstream effect, activating JAK kinases and STAT3 transcription factors [11]. Cytokines and chemokines that regulate angiogenesis are also regulated by these pathways which may impact intestinal recovery following ischemia. Interferon-inducible protein 10 (IP-10), also known as C-X-C motif chemokine 10 (CXCL10), is a member of the chemokine CXC family. Previous studies have shown that IP-10 can inhibit angiogenesis through the JAK-STAT signaling pathway *in vitro* and *in vivo* [12–15]. Conversely, IL6 has also been shown to stimulate VEGF production in other tissues [16, 17], which may help drive the proangiogenic pathway following tissue injury. The balance of IL6 signaling in the intestine likely plays a key role in intestinal repair following injury. Therefore, we hypothesized that: 1) Intraperitoneal injection of human IL6 after ischemia would increase intestinal perfusion 2) decrease mucosal injury scores, and 3) alter VEGF and IP-10 levels in the intestinal tissue.

MATERIALS AND METHODS

Human IL-6

Vials of recombinant human IL-6 of 5μL and 25 μL (Sino Biological) were reconstituted with double distilled-water on a 1:10 dilution, aliquoted in 1.5 mL tubes for each treatment dose (10 with 20ng, 10 with 200 ng and 10 with 2μg) and stored at −20C. Before use, the reconstituted IL-6 was thawed in the refrigerator and used the same day.

Murine ischemia/reperfusion (I/R) model

The protocol and use of animals were approved by the Indiana University Institutional Animal Care and Use Committee. Adult C57BL/6 male mice between 8 to 12 weeks of age and 20 to 30 grams were used (Jackson Labs, Bar Harbor, ME). Mice were acclimated to their environment for at least 48 hours before any experimentation and were maintained in a 12-hour light-dark cycle with free access to chow and water. Before the procedure, 3% isoflurane was used for induction anesthesia. Animals were then placed on a heated blanket to maintain body temperature and remained anesthetized with 1.5% isoflurane. Abdomens were shaved and prepped with 70% ethanol and betadine. One milliliter of 0.9% normal saline was injected subcutaneously and a midline laparotomy performed. Intestines were eviscerated to visualize the small bowel mesentery. Using an atraumatic microvascular clamp, the mesenteric root was occluded for 60 minutes. Laser Doppler imaging (LDI) (Moor instruments, Wilmington, DE) was used to take images before and after clamping the mesenteric vasculature to ensure the arterial occlusion was effective. During the 60 minutes of arterial occlusion, intestinal tissue was reintroduced to the abdominal cavity and closed with silk suture to prevent heat and water loss. After the 60 minutes, abdominal cavities were re-opened, and the clamp removed. Before closing the peritoneum, mice received the treatment; 250 μL of PBS or 250 μL of PBS containing 20ng, 200ng or 2 μg of IL6. Subcutaneous buprenorphine (1 mg/Kg) and carprofen (5mg/Kg) were used for analgesia. Animals were continuously monitored while recovering from anesthesia, then placed back in their cages for recovery.

After 24 hours, animals were re-anesthetized, and abdomens were reopened. Small bowel and mesentery were exposed to measure perfusion with LDI. For each mouse, 100% of perfusion was represented by their baseline (n=9/10 mice per group). Animals were then euthanized by cervical dislocation and tissue segments were harvested for histologic and cytokine analysis.

Histology

Terminal ileum explants were fixated in 4% paraformaldehyde, subsequently dehydrated in 70% ethanol, and embedded in paraffin to be cut on the microtome. Segments were placed on a slide and stained with hematoxylin-eosin stain (H&E). A well-defined histologic scoring system was then used to determine the tissue injury: 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 tip; 4, loss of more than half of the villous structure; 5, transmural necrosis[18, 19]. (n=9/10 intestinal segments per group).

Cytokines

Intestinal tissue was harvested immediately following euthanasia, was snap frozen in liquid nitrogen, and stored at −80C. For protein homogenization, tissue was thawed and handled in a 4C cold room. Eppendorf tubes (1.5 mL) containing stainless steel beads, 400 μL of RIPA buffer with protease inhibitor and phosphatase inhibitor (1:100 dilution, Sigma) were used for homogenization. Homogenates were then centrifuged at 12.000 rpm for 5 minutes and supernatant was transferred to new Eppendorf tubes for storage at −80C. Total protein was quantified by Bradford Assay using a spectrophotometer (Versamax microplate reader, Molecular Devices). IP-10 and VEGF levels in intestinal tissues were measured by ELISA (R&D systems). Experiments were performed in duplicate, and were repeated to verify results (n= 9/10 samples per group).

Statistical analysis

Perfusion and cytokine data were analyzed and deemed normally distributed. One-Way Anova and t-test were used to compare groups. Perfusion results were expressed as mean of the percentage of recovery compared to baseline (mean% ± SEM), while cytokine data was expressed as nanograms of cytokine per gram of tissue sample (mean± SEM). Histologic injury data was not normally distributed; therefore, groups were compared using Kruskal-Wallis and Mann-Whitney tests when appropriate. Histology injury results were expressed as the median with 25%−75% interquartile range (IQR). A $p=< 0.05$ was considered statistically significant.

RESULTS

Post-ischemic perfusion

After comparing perfusion measurements with the baseline, the low dose IL6 group showed a higher percentage of recovery when compared to the vehicle group (Vehicle: 45.37±8.79%; Low dose IL6: 69.12±4.93%, respectively, $p=<0.05$, Figure 2). No difference in perfusion was found between the medium dose group or the high dose group when

compared to vehicle. Low and medium dose IL6 groups maintained better perfusion than the high dose IL6 group ($p<0.05$)

Mucosal injury

Both IL6 low and medium dose groups showed decreased mucosal injury scores (Vehicle =3, IQR=2.25; Low dose=2, IQR=1; Medium dose=1, IQR= 0,) compared to vehicle ($p=<0.05$). Vehicle and high dose groups showed similar injury scores (Vehicle =3, IQR=2.25 and IL6 High Dose=3, IQR=2 respectively, $p=<0.05$, Figure 3) Low and medium dose IL6 therapy appeared to decrease intestinal mucosal injury. However, higher doses did not appear to provide any beneficial effects. High dose IL6 provided higher injury scores than either low dose or medium dose IL6 ($p<0.05$).

Intestinal VEGF and IP-10

Intestinal levels of VEGF were higher in low dose IL6 treatment groups (97.7±17.32) compared to vehicle (50±5.16, $p<0.05$, Figure 4A). There were no significant differences in VEGF levels between Vehicle, and medium or high IL6 groups. IP-10 levels were lower in low dose and medium dose groups (77.8±12.5; 75±17.97 respectively) when compared to vehicle and high dose groups (142.1±25.55; 163.7±27.63 respectively, $p<0.05$, Figure 4B).

DISCUSSION

The management of patients with intestinal ischemia is challenging. Current therapies involve surgical resection of necrotic tissue to mitigate the systemic inflammatory response, organ failure, and death. New therapies, where the intestinal tissue can be protected against damage or repaired before irreversible injury occurs would be beneficial. In our study, we demonstrated that lower doses of IL6 therapy immediately following intestinal ischemia could improve post-ischemic perfusion and intestinal injury following ischemia. However, higher doses do not appear to be beneficial.

The results of this study imply an important balance between the classic and alternate (anti-inflammatory vs pro-inflammatory respectively) pathways of IL6 signaling. Lower doses of IL6 likely work to stimulate the classic pathway which may work to improve outcomes. However, once this pathway is saturated, the alternate pathway likely becomes activated with higher doses of IL6. This certainly makes sense as higher doses of IL6 have been shown to induce the inflammatory cascade and the acute phase response [20–23].

IP-10 levels in the intestinal tissue were lower in both low dose and medium dose IL6 treatment groups compared to the high dose and vehicle groups. IP-10 is a chemokine involved in recruiting leukocytes to the site of inflammation [24]. In addition, and likely more important, several studies have shown that IP-10 can inhibit angiogenesis through the JAK/STAT signaling pathway. Therefore, an observation that IP-10 was downregulated suggests that the local inflammatory response associated with leucocyte infiltration, as well as the signals to inhibit angiogenesis, were also downregulated following lower dose IL6 therapy. However, when higher doses of IL-6 were used, IP-10 levels were significantly elevated compared to low and medium dose groups but exhibited no difference above

vehicle. This may suggest that higher doses of IL6 provide no effect above non-treated animals.

VEGF, another potent factor that contributes to angiogenesis, was significantly higher in the low dose treatment group compared to vehicle. VEGF and IP-10 may function as one of multiple balances for angiogenesis through the JAK/STAT pathway. Therefore, it is possible that the IL-6 pathway functions as a fulcrum to regulate this balance. Lower levels of IL6 tip the scale to lower IP-10, higher VEGF, more angiogenesis, and better outcomes, while higher levels of the drug tip the scale to higher IP-10, lower VEGF, lower post-ischemic angiogenesis, and worse recovery.

It is unclear in this study if IL6 promoted angiogenesis in our *invivo* model or simply worked to improve perfusion within the already present mesenteric vascular bed. *Ex vivo* studies utilizing pressure myography to assess vascular tone previously showed no vasodilation with direct application of IL6 to the bath chamber. However, when IL6 was applied during a similar *in vivo* model, significant vascular dilation was noted. This suggested that IL6 required significant interaction with parenchymal or intravascular factors in order to promote arterial vasodilation [25]. Other studies have shown that IL6 can directly induce vessel sprouting and can work to promote endothelial cell proliferation and migration [26]. Given that IL6 signaling also stimulates VEGF production it is likely that angiogenesis plays a significant role in intestinal recovery following injury. Further studies are needed to determine the mechanism in which IL6 promotes recovery of mesenteric perfusion and intestinal architecture following injury. Utilizing models that allow for longer recovery periods could potentially help answer these questions.

LIMITATIONS

A few limitations exist that should be mentioned for this study. One limitation to the study is that the SMA ligation model of intestinal I/R does not model clinical intestinal ischemia to its fullest. Although complete small bowel ischemia is possible secondary to SMA thrombus or volvulus, the majority of clinically relevant intestinal ischemic episodes are due to segmental intestinal ischemia, such as may be seen with incarcerated hernias. Nonetheless, the SMA ligation model mimics the most severe form of intestinal ischemia, and therefore, is likely the best animal model available to test the effectiveness of new therapies.

A subsequent limitation surrounds the application of intraperitoneal IL6 therapy via an intraperitoneal route. Although an intravenous route of administration is likely more ideal, the application of IL6 directly to the bowel after ischemic injury allows for local treatment directly to the intestine and the mesentery during a situation in which perfusion to the bowel is likely severely compromised.

CONCLUSION

The mechanism involved in the modulation of the inflammatory response by IL6 is complex. Our study suggests that lower doses of intra-peritoneal IL6 after intestinal ischemia improves perfusion, decreases intestinal injury, and modulates the production of VEGF and IP-10 in intestinal tissue. However, with high doses of IL6, the benefits of therapy are lost.

These findings would suggest a delicate balance between the classic and alternative arms of the IL6 signaling cascade. Further studies are necessary to elucidate the mechanisms involved in such protection prior to clinical implementation.

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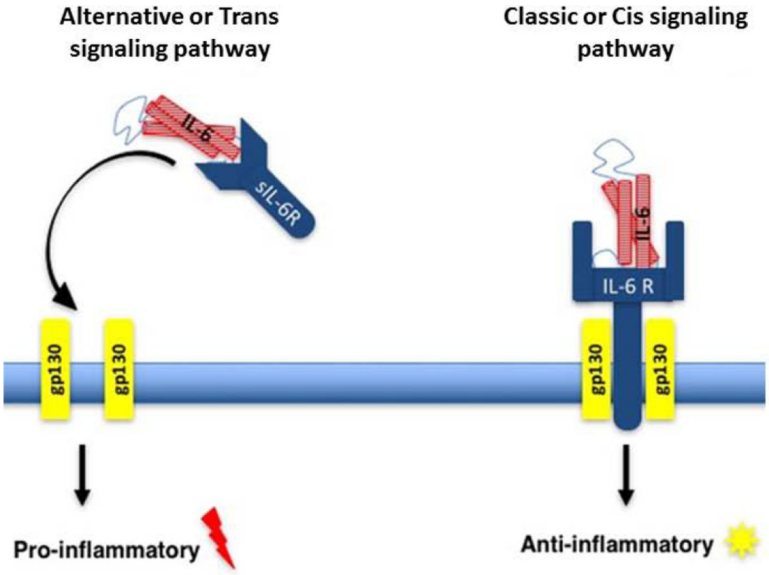

Figure 1- Mechanism of IL-6: Proinflammatory and anti-inflammatory actions of IL-6.
Image shows both anti-inflammatory (classic) or cis pathway on the right (membrane bound IL-6 receptor) and pro-inflammatory (alternative) or trans pathway on the left (soluble IL-6 receptor). Both pathways require two molecules of gp130.

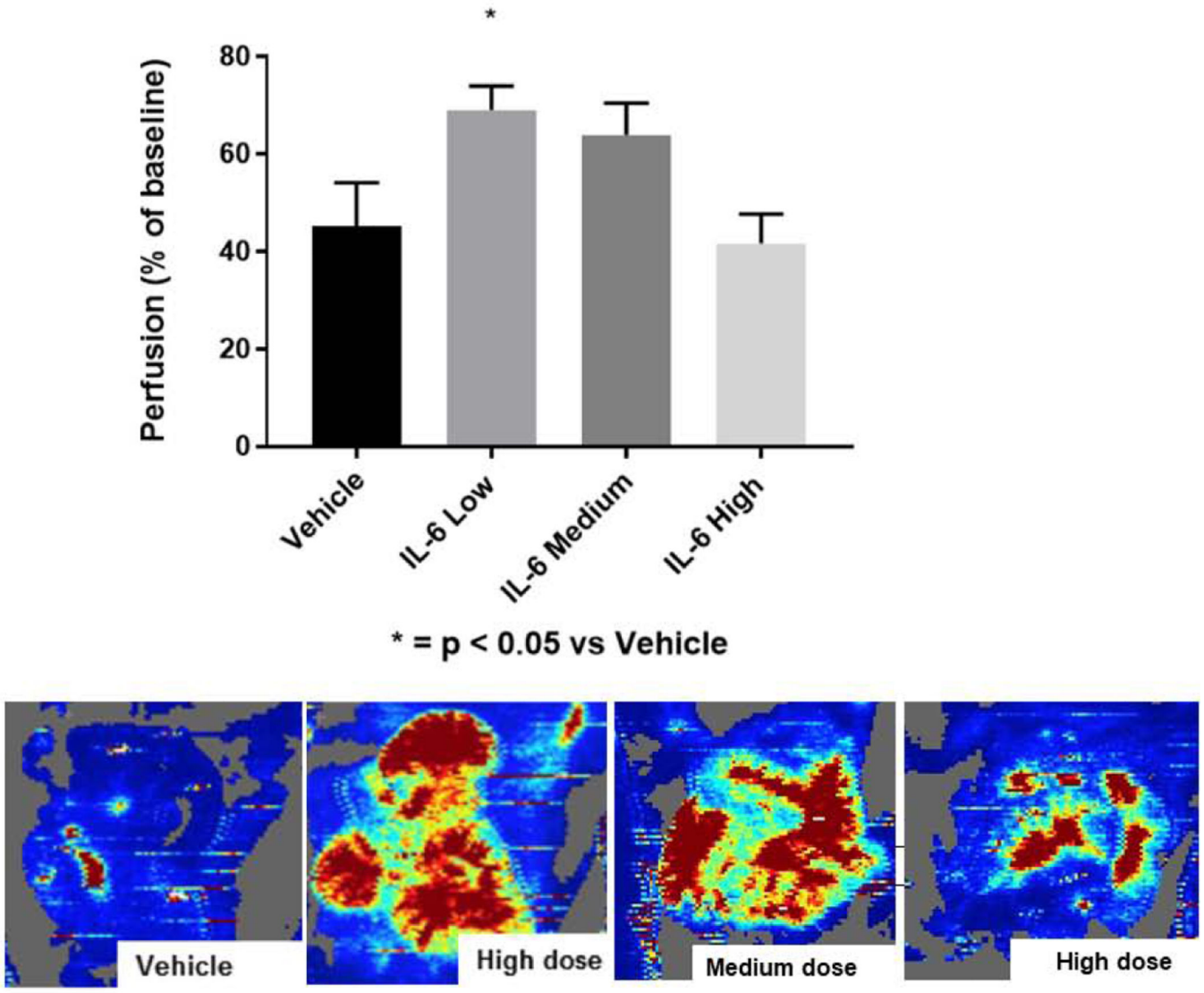

Figure 2- Mesenteric perfusion:

Low dose IL6 was able to improve mesenteric perfusion above vehicle. Medium and high doses of IL6 did not have an effect on perfusion above vehicle. (*=p<0.05 vs. vehicle, #=p<0.05 vs. IL6 High).

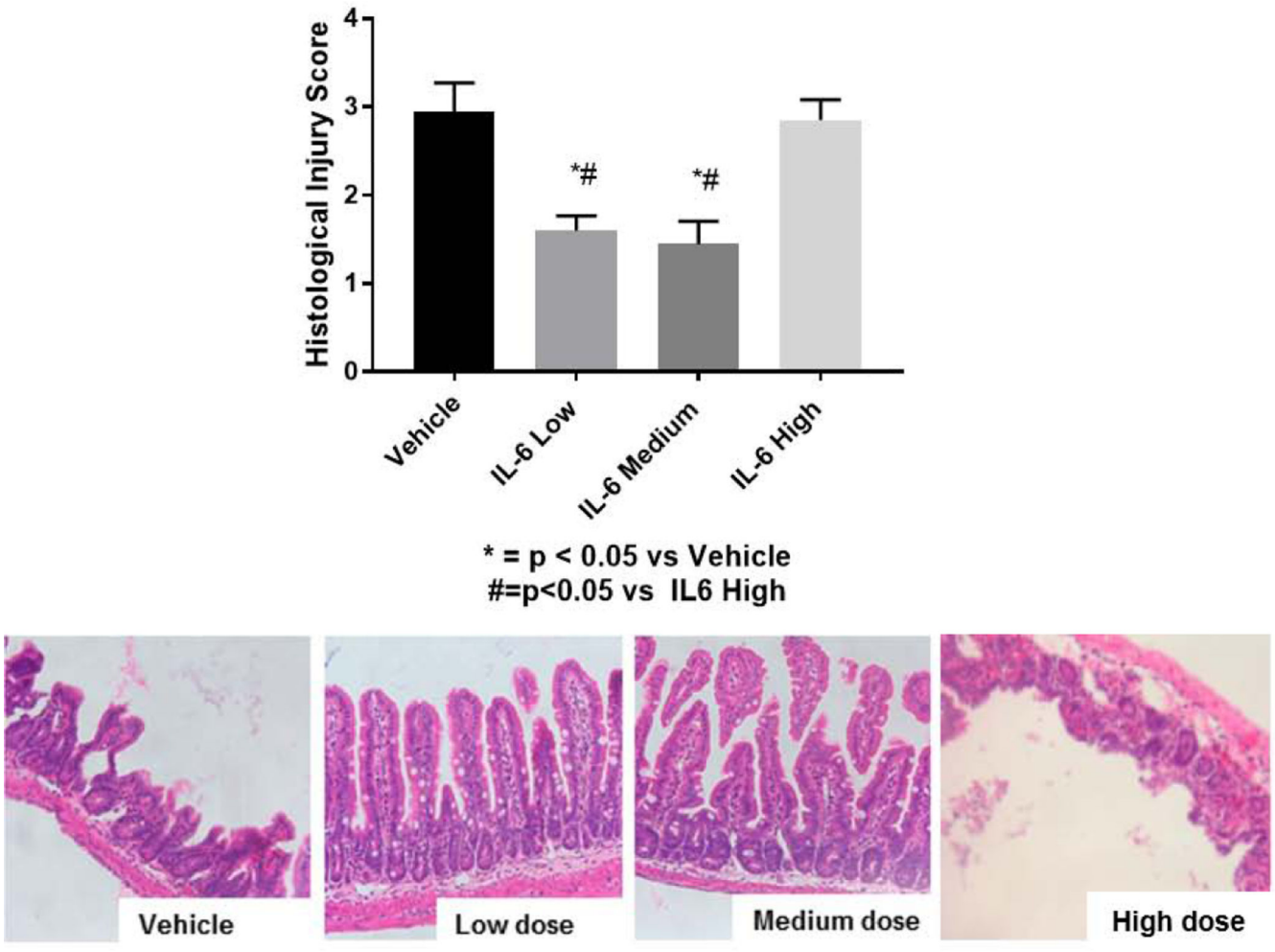

Figure 3- Mucosal injury:

Low and medium doses of IL-6 improved mucosal injury scores compared to vehicle. High dose IL6 was similar to vehicle, (*=p<0.05 vs. vehicle, #=p<0.05 vs. IL6 High)

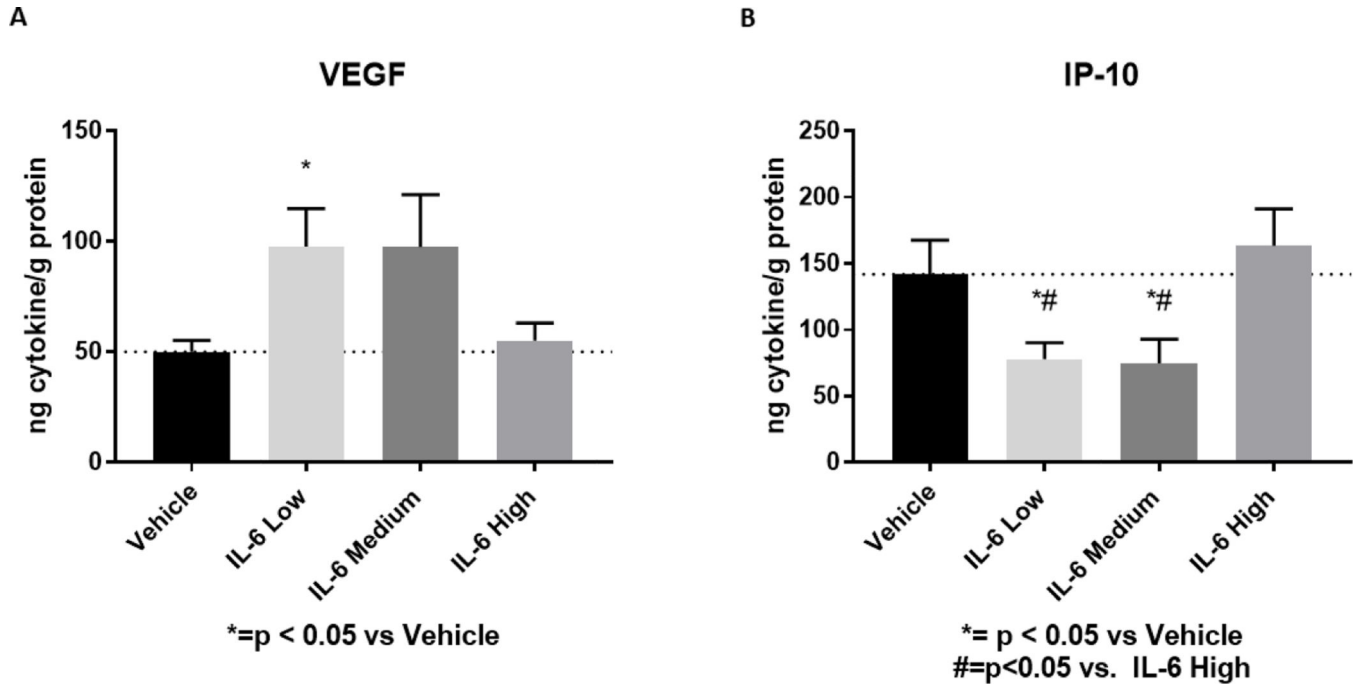

Figure 4- Intestinal cytokines:

(A) Levels of VEGF were elevated in the low dose IL6 group when compared to vehicle (*=p<0.05 vs. Vehicle). **(B)** IP-10 levels were elevated in vehicle and high dose groups when compared to low dose and medium dose IL6 groups (*=p<0.05 vs. Vehicle, #=p<0.05 vs. IL6 High).