

Iloprost reduces colonic injury in ischemic colitis in rats¹

Iloprosta reduz o dano colônico na colite isquêmica em ratos

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ABSTRACT

Purpose: Evaluate the effects of iloprost administration in the early period of ischemic colitis and the mechanism that how these effects develop. **Methods:** Thirty two Wistar albino female rats with an average weight of 220g were divided into four groups of eight rats. In group 1 the rats were given iloprost and sacrificed after 24 hours and in group 2 they were sacrificed after 24 hours without any iloprost. The rats in group 3 were administrated iloprost and sacrificed after 72 hours and in group 4 they were sacrificed at 72th hour without iloprost. The differences between the groups as tissue damage, vascularization or apoptosis were assessed statistically. **Results:** Oxidative damage and apoptosis were less pronounced and vascularization was better developed in rats that were given iloprost and sacrificed at 24th hour later in contrast to the rats that were not treated with iloprost. But there was no statistical difference among the groups at 72th hour. **Conclusion:** Iloprost inhibited leucocyte infiltration, decreased proinflammatory cytokines and enhanced angiogenesis so that the oxidative stress and inflammatory response decreased resulting in lesser tissue damage.

Key words: Colitis, Ischemic. Iloprost. Epoprostenol. Arachidonic Acid. Prostaglandins. Rats.

RESUMO

Objetivo: Avaliar os efeitos da administração de iloprosta no período precoce da colite isquêmica e o mecanismo da evolução destes efeitos. **Métodos:** Trinta e dois ratos Wistar fêmeas em torno de 220g foram distribuídos em quatro grupos de oito ratos. No grupo 1 administração de iloprosta e sacrificados após 24 horas; no grupo 2 foram sacrificados após 24 horas sem iloprosta; no grupo 3 foi administrado iloprosta e sacrificados após 72 horas; no grupo 4 foram sacrificados após 72 horas sem Iloprosta. As diferenças entre os grupos no referente a dano tecidual. vascularização ou apoptose foi apurada estatisticamente. **Resultados:** Dano oxidativo e apoptose foram menos acentuados e a vascularização foi melhor nos ratos que receberam iloprosta e sacrificados após 24 horas em contraste com os ratos que não receberam iloprosta. Porém, não houve diferença estatisticamente significativa entre os grupos de 72 horas. **Conclusão:** Iloprosta inibe infiltração leucocitária, diminui a ação inflamatória de citocinas e estimula angiogênese resultando em menor dano tecidual.

Descritores: Colite Isquêmica. Iloprosta. Epoprostenol. Ácido Araquidônico. Prostaglandinas. Ratos.

Introduction

Ischemic colitis is the tissue damage and inflammation that occurs as a result of insufficient blood supply to the colon and it was reported for the first time by Boley *et al.*¹ almost 90% of the cases are 60 years or older but it can also occur in younger people. Since it may be self limiting and the diagnosis is usually made by suspicion the exact incidence can not be estimated. The insufficient blood supply may be due systemic circulatory abnormalities that induce hypo-perfusion such as congestive heart failure or shock but it may also due to local factors like mesenteric vasoconstriction or obstruction as well. The clinical picture may vary from abdominal distention to acute abdomen. Mild cases may respond to bowel rest, intravenous hydration and antibiotics but severe cases may result in intestinal gangrene, perforation and sepsis. There is yet no effective treatment modality neither in ischemic nor ischemia-prone cases.

Iloprost is a synthetic stable long acting prostacyclin (PGI₂) analogue that exerts its effects in the endothelium. Within normal blood pressure range it has a selective mesenteric vasodilator effect². The half life of iloprost is 4 minutes and it has an elimination duration of 30 minutes. It decreases the thrombocyte aggregation, leukocyte activation and chemotaxis and suppresses the superoxide anion production. It is also a potent vasodilator. Manasia *et al.*³ reported that low dose (0.075µg/kg/min) iloprost increased the superior mesenteric blood flow in a systemic hypotension model caused by septic shock. Iloprost was also reported to have liver and kidney sparing properties in ischemia-reperfusion damage^{4,5}. In patients with critical limb ischemia iloprost increased the endothelial progenitor cells in vivo and induced angiogenesis⁶. Iloprost was also reported by Lehman *et al.*⁷ that it has a protective effect against endotoxin induced intestinal damage in rats and that was attributed to the decrease in leucocyte adhesion in the circulation and decrease in oxidative damage by inhibition of proinflammatory cytokines such as TNF- α and IL-6^{8,9}.

It is also reported that prostacyclin has stimulatory effects on angiogenesis¹⁰. The angiogenic function of the human endothelial progenitor cells (EPG) is closely associated with the arachidonic acid metabolism and prostacyclin biosynthesis. The in vivo and in vitro angiogenic capacity of EPG was shown to be related with the endogenous prostacyclin levels produced by EPG¹¹.

Iloprost is reported in the literature as it has protective effects on tissue damage by the way that it induces angiogenesis and is cytoprotective, increases intestinal blood flow and diminishes neutrophil aggregation. But there is no study yet that

explored its clinical or experimental effects on ischemic colitis. We aimed to study the effects and the mechanisms of action of the early administration of iloprost on ischemic colitis.

Methods

The study was approved by the ethical committee for the research on the animals and run in the laboratories for experimental research in Istanbul University between October 2009 and January 2010.

Thirty two Wistar albino female rats with an average weight of 220g were used in the study. They were kept in individual cages for 12 hours day and 12 hours night pattern and were fed normal diet in the pre-and postoperative period.

The rats were randomized into 4 groups so that each group has 8 rats and ischemic colon was formed in each rat.

Group 1: rats were given iloprost and were sacrificed at 24th hour.

Group 2: rats were sacrificed at 24th hour but not given iloprost.

Group 3: rats were given iloprost and were sacrificed at 72th hour.

Group 4: rats were sacrificed at 72th hour but not given iloprost.

The rats were put under anesthesia by giving 10mg/kg ketamine injection intraperitoneally. The incision site was shaved and cleaned by 10% povidine-iodine solution. Following laparotomy the ischemic colon was formed by ligating and cutting the vessels in the mesenteric tissue supplying about 2 to 4 cm. area of the distal colon.

Rats in group 1 and 2 were subjected to 2µg/kg iloprost (Iloprost 20µg/ml; Schering, Berlin) by intravenous infusion.

The rats in groups 1 and 2 were sacrificed at 24th hour and the rats in groups 3 and 4 were sacrificed at 72th hour. Tissue was sampled from the experimental ischemic colon for biochemical and histopathological study.

Iloprost therapy

The treatment dosage of the iloprost in the treatment of ischemic colitis is not present in the literature. The dosage was calculated by reviewing literature about the amount of iloprost that was effective on the ischemic tissue and the amount was given as a single dose after the surgery iloprost was administrated in the early period after the laparotomy to the rats in groups 1 and 3^{12,13}.

The assessment of the oxidative damage

Tissue samples were fragmented to small pieces homogenized by 1/5 (W/V) phosphate buffered saline solution. The values were measured by Shimada 1601 UV/VIs spectrophotometry (Shimada, Japan) using Lowry and Rosenbraugh method. The protein concentration was calculated according to the Lowry method.

The measurement of malondialdehyde (MDA)

The measurement of MDA was performed by tributyrac acid method. 1µmol/L Ferrumsulphate at pH 2.0 was added to the solution and the absorption was recorded at lightwave 535nm. The normal value of MDA in the tissue was accepted as <1.0µmol/L.

The measurement of nitric-oxide (NO)

NO was calculated by the calorimetric method (Boehringer Mannheim Kit; Kat no: 01756281). The normal value for NO within tissues was accepted as <0.5pg/mg.

The measurement of vascular endothelial growth factor (VEGF)

The plates coated with antipoliclonal animal VEGF were used and the tests were performed by automatic Best 2000 (Biokit) microelisa. The normal value of VEGF in the tissue was accepted <50pg/mL/mg.

The measurements of tumor necrosis factor –alpha (TNFα) and interleukin-6 (IL6)

The levels of TNFα ang IL6 within the homogenate were calculated by ELISA (Quantikene-Sensitivity-Human by RPD systems, USA). The normal value of TNFα was accepted as <3.5ng/mg and of IL6 as <3.0pg/mg.

Histopathological assessments

The tissue samples were fixed in 10% formaldehyde and satined by hematoxyline-eosine for microscopic examineion under 20x and 100x magnification. The healing process and inflammatory response as well as granulocyte and fibroblast infiltration, necrosis, exudate formation and new capillary generation were assessed semiquantitavely. The results were

scored from 0 to 3¹⁴.

- 0 No change
- 1 Mild changes
- 2 Moderate changes
- 3 Dense changes

Statistical evaluation

The results were assessed as mean and standard deviation. The data for the groups were analyzed by Mann Whitney-U tests and the multiple variable data between the groups were analyzed by post-hoc test (Tukey’s HSD test). P<0.05 was accepted as statistically significant. General assessment of the data was performed by the computer program Statistical Package for the Social Sciences for Windows (SPSS for windows 11.5. SPSS, Chicago, Il; USA).

Results

No mortality occurred among the study animals. The rats were sacrificed 24 and 72 hours after the performance of colonic ischemia. Biochemical values and comparisions were given as tables.

When biochemical data were analyzed the differences in the levels of TNFα and IL6 were found to be statistically significant (p=0.029 for both). MDA and NO levels as the important parameters of oxidative damage were significantly different in group 1 in contrast to group 2 (p=0.001 and p=0.002). Also the levels of VEGF at 24th hour was significant in group 1 in contrast to group 2 (Table 1).

TABLE 1 - Statistical comparision of the data between groups 1 and 2.

	GROUP 1		GROUP 2		P
	MEAN	SD	MEAN	SD	
IL 6	0,627	0,147	0,984	0,123	0,029
TNF alfa	0,530	0,089	0,869	0,104	0,029
NO	101,171	3,396	135,625	5,181	0,001
MDA	1,753	0,227	2,300	0,379	0,002
VEGF	2,864	0,312	2,014	0,382	0,002
CAS	22,929	1,533	30,445	1,463	0,002

The level of caspase-3, an indicator of apoptosis, was found to be lower at 24th hour in group 1 compared to group 2 (p=0.002). But there were no statistically significant difference in the caspase-3, IL6, MDA or VEGF levels at 72th hour (p=0.620, p=0.06, p=0.65, p=0.08). TNF α and NO levels were lower in group 3 at 72th hour (p=0.04, p=0.001) (Table 2).

TABLE 2 - Statistical comparison of the data between groups 3 and 4.

	GROUP 4		GROUP 3		P
	MEAN	SD.	MEAN	SD.	
IL 6	1,087	0,117	1,047	0,147	0,06
TNF alfa	1,346	0,210	0,530	0,089	0,04
NO	149,286	9,499	101,171	3,396	0,001
MDA	2,743	0,196	2,353	0,227	0,65
VEGF	2,345	0,432	2,554	0,513	0,620
CAS	35,741	0,848	34,634	1,533	0,08

Histopathological results

The tissues were analyzed by an independent blinded pathologist. Epithelization, monocyte and neutrophil infiltration, necrosis and capillary proliferation were assessed. All the parameters were statistically higher in group 1 compared to group 2 (monocyte infiltration p=0.020, neutrophil infiltration p=0.42, capillary proliferation p=0.001) (Figures 1 and 2) (Table 3).

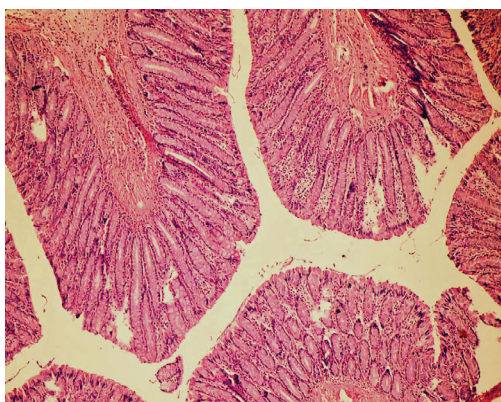


FIGURE 1 - Leucocyte and lymphocyte infiltration in mucosa and submucosa in ischemic colitis (Hematoxyline-eosin staining X100 magnification).

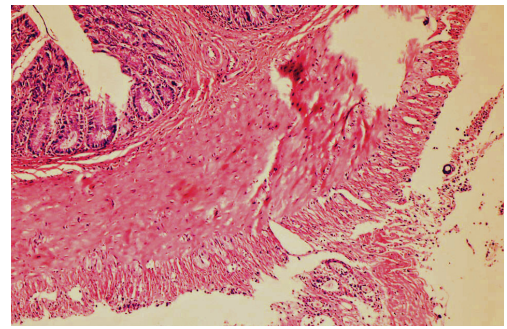


FIGURE 2 - Leucocyte and lymphocyte infiltration in mucosa and submucosa in ischemic colitis terated with iloprost (Hematoxyline-eosin staining X100 magnification).

TABLE 3 - Statistical comparison of histopathological data between groups 1 and 2.

Early sacrificed group	Group 1		Group 2		P
	MEAN	SD.	MEAN	SD.	
Neutrophil infiltration	2,75	+/- 0,46	1,875	+/- 0,64	0.042
Monocyte infiltration	2,5	+/- 0,75	1,375	+/- 0,74	0.020
Epithelization	0,75	+/- 0,46	1,125	+/- 0,35	0.065
Necrosis	1,62		1,125	+/- 0,64	0.089
Capillary proliferation	3,125	+/- 0,64	2,5	+/- 0,75	0.001

But the results at 72th hour did not show any significance (p>0.05)

Discussion

Systemic or local factors that cause decreased intestinal blood circulation may result in ischemic colitis, a life threatening intestinal inflammation and damage. It is most frequently observed in the colon. In contrast to small bowel ischemia there is rarely a major arterial or venous obstruction in colonic ischemia which is usually accompanied by small vessel pathology or diminished blood flow. Vascular disorders, diabetes mellitus, vasculitis and hypotension may play role in the etiology. Ligation of inferior mesenteric artery during abdominal aortic surgery or in colorectal surgery increases the predisposition of the colon to ischemia. Thrombosis or emboli can also result in ischemia.

Ischemic colitis is more frequent on the splenic flexura area of the colon and the treatment depends on the clinical presentation. Colonic ischemia usually respond to the medical therapy compared to the small bowell counterpart. A report from Madrid concluded that there was a hundred percent viability in the

colonic mucosa if therapy started early among the 21 patients with superior mesenteric artery embolism but the viability decreased by postponed therapy to 56% after 12 hours and to 18% after 24 hours. This study strongly recommends the early start of the therapy¹⁵. Bowel rest and broad spectrum antibiotics are among the mainstays of the therapy and 80% of the patients recover eventually. If the hypotension is the cause of the clinical picture then it should rapidly treated to the normal hemodynamic parameters. With no response to the medical therapy after 2-3 days or should the clinical picture of the patient deteriorates then surgical exploration is indicated. Mortality may be 50 to 80% even with the best treatment. The recovery of the oxygen supply to the ischemic colon is the main aim during the therapy. In addition to the normal vascular supply promotion of the angiogenesis to the ischemic wound healing is necessary and can be observed by the levels of the VEGF in the molecular level. Di Stefano *et al.*⁶ reported that iloprost increased the levels of VEGF and promoted the angiogenesis in vivo so that the progenitor endothelial cell number expanded and ischemic limbs showed clinical improvement. We found that early administration of iloprost enhanced VEGF production in the tissue and there was a statistically significant increase compared to the control group (group 1 with iloprost therapy after ischemia vs. group 2 without iloprost therapy) ($p=0.002$) but there was no difference after 72 hours ($p=0.6$). Histopathologically increased levels of vascularization was observed in group 1 at 24th hour that and although the increase was sustained even at 72 hours it was not statistically significant.

When the ischemia advances the mediators are secreted at the cellular level that result in tissue injury and oxidative damage. The cells proceed to the apoptosis which is a programmed cellular death required for physiologic homeostasis¹⁶. Bortoletto *et al.*¹⁷ claimed that ischemia increases apoptosis during intestinal ischemia and hyperbaric oxygen therapy can protect the mucosa against the ischemia-reperfusion injury. Similarly Aslan *et al* reported that there is increased inflammation and apoptosis in ischemic bowel anastomoses and mesalamine (5-ASA) administration decreased the inflammation in the ischemic area by suppressing the free oxygen radicals¹⁸. We found too that inflammation, oxidative damage and apoptosis is increased in ischemia produced rats. To survey the level of apoptosis at the tissue level the caspase-3 levels were assessed by biochemical methods. The caspase family activates a broad spectrum of genes that play a role in the synthesis of proinflammatory cytokines and acute phase proteins. In addition caspase-3 activity can use as a measure of apoptosis¹⁹. The caspase-3 activity was significantly different in iloprost given group 1 at 24th hour compared to the

control group 2 but this difference disappeared at 72th hour. This can be translated that iloprost decreases the apoptosis in the early period of ischemia. Apoptosis is a complex process but we failed to prove the exact mechanism how the iloprost produced anti-apoptotic activity. The anti-inflammatory mechanism, increased vascularization or antioxidative protection all may play a role. Manasia *et al.*³ showed that administration of 0.18 μ g/kg/min iloprost to the pigs subjected to septic shock increased the mesenteric perfusion without affecting mean arterial pressure by increasing cardiac output and increasing superior mesenteric artery blood flow. The same study also gave the information that the inflammation produced by endotoxic shock was diminished with the use of iloprost. We found histopathologically that inflammatory cell activation was lower and enhancement of vascularization was better in the iloprost group. The results were supported by biochemical survey and early administration of iloprost was proven even to be further effective.

Iloprost is long acting stable prostacycline (PGI₂) analogue. PGI₂ is an endothelial cyclooxygenase function product that decreases thrombocyte aggregation, leucocyte activation, chemotaxis and superoxide anion production. It is a potent vasodilator²⁰ by relaxing the vascular tone it prevents vascular obstruction and helps the vessels carry more blood to the tissue. Iloprost decreases the blood viscosity, dissolves plugs and prevents aggregation of shed off endothelial cells^{21,22}. Prostaglandins enhance the blood flow in ischemia-reperfusion injury in skeletal muscles²². Iloprost had therapeutic effects compared to placebo administration in patients with diabetic feet ulcers²³⁻²⁵. The patients complained lesser pain and improved ulcers with clinically proven decrease in the risk of amputation both during the therapy and also in the 3-6 month follow-up period after the treatment²⁶. Iloprost and propentoxiphylline reduces the macrophage produced IL6 and TNF α ²⁷⁻³². It coordinates the production of genes that control cellular growth and inflammation and decreases productions of cytokines in vitro. In addition to its vasodilator and antiaggregating effects, it may also has anti-inflammatory properties³³. Iloprost can be administered as through inhalational route or by intravenous infusion³⁴. The requisite for infusion makes patient compliance difficult and inpatient treatment is required^{34,35}. We introduced iloprost intraperitoneally at a dose of 2 μ g/kg/min since it is very difficult to infuse the drug intravenously for a long time in rats. K. Vasiliadis also used the drug intraperitoneally at the same dosage to counteract the side effects of the chemotherapeutic agents and to improve the healing of the colonic anastomoses¹². We had set forth the study by reviewing the literature for the beneficial effects of iloprost and conclude that it has diminished

the oxidative damage, decreased proinflammatory cytokines but enhanced angiogenesis so that it has especially prevented the ischemic damage in the early period histopathologically. The main shortfall of the study is that we could not identify the mechanisms by which the iloprost produced its protective effects.

Conclusions

Oxidative damage, apoptosis and increased myeloperoxidase (MPO) activity contributed to tissue damage in ischemic colon. Additionally increased IL6, NO, caspase-3 and MDA and TNF α levels were interrelated with the ischemic injury. Iloprost was found to have protective properties if administered in the early period of ischemic colitis. It inhibited leucocyte infiltration, decreased proinflammatory cytokines and enhanced angiogenesis so that the oxidative stress and inflammatory response decreased resulting in lesser tissue damage.

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