

## Trace element levels in ischemia-reperfusion injury after left colonic anastomosis in rats and effects of papaverine and pentoxifylline on vascular endothelial growth factor in anastomosis healing

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### Abstract

**Background and study aims :** Due to their high morbidity and mortality, anastomotic leakage and disruption are still serious problems in colonic surgery. Bowel clamps applied during anastomosis in order to prevent abdominal contamination with colonic contents, may cause microcirculation and perfusion problems and subsequent ischemia-reperfusion injury. Papaverine, a myorelaxant and vasodilator, and pentoxifylline, a hemorrheologic agent are used for microcirculation disorders and vascular endothelial growth factor (VEGF) is a stimulator of angiogenesis. With this experimental study, we aimed to measure trace element [copper (Cu) and zinc (Zn)] levels in ischemia-reperfusion injury due to clamps after left colonic anastomosis in rats and show the effects of papaverine and pentoxifylline on VEGF that stimulates angiogenesis in anastomotic healing.

**Materials and methods :** 50 female Wistar albino rats were randomized in 5 groups (n : 10). Laparotomy in group 1, left colonic transection and anastomosis in group 2, and clamp application 1 cm proximal and distal to the anastomosis (for about 20 minutes long) during left colonic transection and anastomosis in groups 3, 4 and 5 were performed. Additionally, after the operations, pentoxifylline (Group 4) and papaverin (Group 5) were injected intraperitoneally. On the 10<sup>th</sup> postoperative day, plasma trace element and plasma VEGF levels were measured.

**Results :** In this study, VEGF levels in group 1 were significantly low and this was explained as being exposed to hypoxic damage less than the other groups. In group 3, VEGF levels were significantly higher showing that the hypoxic stimulus continued without any treatment and in Group 4, significantly lower than Group 3 related to the inhibition of pentoxifylline. Lower VEGF levels in Group 1 were thought to be related to lower VEGF induction due to less hypoxic effect. Zinc, an important trace element of the antioxidant system showed significantly higher levels in Group 4 with pentoxifylline treatment, and this was thought to be related to the antioxidant characteristics of pentoxifylline.

**Conclusions :** During surgical procedures, care should be taken not to cause ischemia to the intestinal tissues, and trace elements that are important in ischemia reperfusion injury should be replaced appropriately. Although the antioxidant effect of pentoxifylline in ischemia reperfusion injury may be beneficial in treatment, its inhibition of VEGF is a disadvantage in wound healing. (*Acta gastroenterol. belg.*, 2011, 74, 22-27).

**Key words :** ischemia-reperfusion injury, colonic anastomosis, trace elements, VEGF, anastomotic leakage.

### Introduction

Colonic surgery has an important place in gastrointestinal tract surgery that currently accounts for a large part of general surgery operations. In colonic surgery, anastomotic leakage and disruption are frequent and serious problems with high morbidity and mortality (1). Most cases of anastomotic leaks occur at the level of the

large intestine. Bowel clamps applied during gastrointestinal anastomosis, in order to prevent intraabdominal contamination with colonic contents, cause microcirculation and perfusion disorders and subsequent ischemia-reperfusion injury. Thus, they cause problems in anastomotic healing as well as systemic processes. It is known that vasoactive agents increase the tissue perfusion, oxygenation and consequently wound healing. The effects of papaverin, an alkaloid which has vasodilatory effect on the vascular smooth muscle (a myorelaxant and vasodilator) and pentoxifylline (a hemorrheologic agent) decreasing the blood viscosity by increasing the deformability of erythrocytes, decreasing the concentration of plasma fibrinogen, and increasing the blood viscosity by inhibiting the adhesion and aggregation of thrombocytes are used for microcirculation disorders (2-4). Furthermore, angiogenesis, an important phase of wound healing, is inevitable in anastomotic healing and vascular endothelial growth factor (VEGF) is one of the most important angiogenesis stimulator. In this study, our aim was to investigate the effects of papaverine and pentoxifylline on VEGF that stimulates angiogenesis, and on trace elements copper (Cu) and zinc (Zn), which have important roles in ischemia-reperfusion injury that occurred as a result of our clamp application during anastomosis.

### Materials and methods

In this experimental study performed in Istanbul University Cerrahpasa Medical Faculty Experimental Medical Research Center, 50 adult, female Wistar-Albino rats weighing between 140-220 g were included. During the study period, the rats were kept under steady environmental conditions and fed on standard laboratory diet and allowed water *ad libitum*. The care of the animals and the approaches and procedures within the study framework were in strict compliance with the principles

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stated by "Guide for the Care and Use of Laboratory Animals" (5). This study was performed in the Experimental Medical Research Center of Istanbul University Cerrahpasa Medical Faculty, and approved by the guidance of Ethics Committee on Animal Research of Istanbul University Cerrahpasa Medical Faculty.

The rats were divided into 5 equal groups (n : 10) and the study plan was as follows :

Group 1 : Sham laparotomy (Control) (C)

Group 2 : Colonic anastomosis (CA)

Group 3 : Colonic anastomosis + Clamp (CA+Cl)

Group 4 : Colonic anastomosis + Clamp + Pentoxiphylline (CA+Cl+PTX)

Group 5 : Colonic anastomosis + Clamp + Papaverine (CA+Cl+PV)

Operations were performed in nonsterile but clean conditions. Anesthesia was carried out by injecting 50 mg/kg ketamine hydrochloride (Ketalar Vial 50 mg – Eczacıbasi) intraperitoneally. While only laparotomy was performed in group 1, a full thickness of a segment of left colon was transected and end-to-end anastomosis was done with 6/0 polypropylene sutures by a single layer technique through a midline abdominal incision in group 2. The incision was closed with 3/0 silk. None of the animals were fed on the first postoperative day. During the anastomotic procedure, clamps (Vascu-Statt single use bulldog clamps) were applied (about 20 minutes) 1 cm proximal and distal to the anastomosis in groups 3, 4 and 5. Including the operation day, 20 mg/kg pentoxiphylline (Trental amp. Hoechst Marion Roussel Drugs) was injected twice a day in group 4, and 100 mg/kg papaverine (Papaverin amp. Tams) was injected once a day in group 5 intraperitoneally for 10 days.

On the 10th postoperative day, intracardiac blood of all the rats were taken via thoracotomy under ether anesthesia, and plasma trace element and plasma VEGF levels were measured. The results obtained were compared and effects of pentoxiphylline and papaverine on anastomotic healing were evaluated according to VEGF levels. Besides, anastomotic colonic segments were resected and bursting pressures were measured.

#### *Cu, Zn and VEGF measurements*

Plasma copper (Cu) (6) and zinc (Zn) (7) levels were measured by atomic absorption spectrophotometer. Atomic absorption (AA) spectrophotometry (8) is used widely in clinical laboratories to measure elements such as aluminum, calcium, copper, lead, lithium, magnesium, zinc, and other metals. Atomic absorption is an emission technique in which an element in the sample is excited and the radiant energy given off measured as the element returns to its lower energy level. However, the element is not appreciably excited in the flame, but is merely dissociated from its chemical bonds (atomized) and placed in an unexcited or ground state (neutral atom). Thus, the atom is at a low energy level in which it is capable of absorbing radiation at a very narrow bandwidth corre-

sponding to its own line spectrum. A hollow cathode lamp with the cathode made of the material to be analyzed is used to produce a wavelength of light specific for the material. When the light from the hollow-cathode lamp enters the flame, some of it is absorbed by the ground-state atoms in the flame, resulting in a net decrease in the intensity of the beam from the lamp. This process is referred to as atomic absorption.

Plasma VEGF levels were measured by ELISA method (Chemicon - cat.no. CYT133).

ELISA (9) is a heterogeneous technique that is widely used in clinical analyses. In this type of assay, one of the reaction components is nonspecifically adsorbed or covalently bound to the surface of a solid phase, such as that of a microtiter well, a magnetic particle, or a plastic bead. This attachment facilitates separation of bound and free labeled reactants. In the most common approach to using the ELISA technique, an aliquot of sample or calibrator containing the antigen to be quantitated is added to and allowed to bind with a solid phase antibody. After washing, enzyme-labeled antibody is added and forms a 'sandwich complex' of solid phase Ab-Ag-Ab-enzyme. Excess (unbound) antibody is then washed away, and enzyme substrate is added, the enzyme catalytically converts the substrate to product(s), the amount of which is proportional to the quantity of antigen in the sample. Antibodies in a sample can also be quantitated using an ELISA procedure in which antigen instead of antibody is bound to a solid phase, and the second reagent is an enzyme-labeled antibody specific for the analyte antibody.

#### *Bursting pressure measurements*

Bursting pressure shows the resistance of the intestinal wall against increasing intraluminal pressure. Although it is lowest in the early phase of the anastomosis on the 2nd and 3rd days, it is equal to that in a normal nonoperated colon on the 7th day. In our study we assessed the effects of chosen agents by measuring the bursting pressures of the anastomosis. After relaparotomy on the 10<sup>th</sup> postoperative day, bursting pressures were measured with a manometer creating constant pressure in the anastomotic bowel using an infusion pump (Perfuser E (type 871112), B. Braun Meisingen A 6 device) at 2 mL/min of NaCl 0.9% and the results were compared.

#### *Statistical analysis*

Measured parameters were expressed as means  $\pm$  Standard Deviation of the mean (SD). The data were examined using Mann-Whitney U test. Differences between groups were considered as statistically significant at  $p < 0.05$ .

## **Results**

In this study, none of the subjects had any wound or intraabdominal infections. Intraabdominal adhesions

Table 1. — Plasma Zn levels

	Zn ( $\mu\text{l/dL}$ )				
	Group 1	Group 2	Group 3	Group 4	Group 5
Mean	8099	8130	8259	9148	6933
Standard deviation	1230	1273	3687	1418	1103

Table 2. — Plasma Cu levels

	Cu ( $\mu\text{l/dL}$ )				
	Group 1	Group 2	Group 3	Group 4	Group 5
Mean	2653	3598	4753	5290	4224
Standard deviation	626	883	1390	2257	936

Table 3. — Plasma VEGF levels

	VEGF (ng/mL)				
	Group 1	Group 2	Group 3	Group 4	Group 5
Mean	3700.8	4318.6	5545.2	4887.4	4915.2
Standard deviation	2396.4	1999.7	2048.6	2073.8	1192.4

were not dense and in all groups dissection was done easily.

*Plasma Zn levels* (Table 1) were found significantly higher in Group 4 according to Group 1, 2, 3 and 5; and significantly lower in Group 5 than Group 1, 2, 3 and 4 ( $p < 0.05$ ). Zn has an important role in antioxidant system and higher results of Zn levels in Group 4 having pentoxiphylline treatment can explain the antioxidant effect of pentoxiphylline.

*Plasma Cu levels* (Table 2) were not found significantly different between groups.

*Plasma VEGF levels* (Table 3) were significantly lower in Group 1 than Groups 2, 3, 4 and 5, significantly higher in Group 2 than Group 1 and significantly lower than Groups 3, 4 and 5. In Group 3, VEGF levels were significantly higher than Groups 1, 2, 4 and 5.

In Group 4 they were significantly higher than Groups 1 and 2 but significantly lower than Group 3. In addition, in Group 5 VEGF levels were significantly higher than Groups 1 and 2 but significantly lower than Group 3.

There were no significant differences between Group 4 and Group 5.

Lower VEGF levels in Group 1 can be related to shorter exposure time to hypoxic injury. However, in Group 3, VEGF levels were higher. This can be attributed to continuous hypoxic stimulus as a result of not having any treatment to decrease ischemia. Significantly higher levels of VEGF in Group 3 than in Group 4 can be related to the the inhibitor effect of pentoxiphylline on VEGF.

*Bursting pressure levels* (Table 4) were significantly lower in Group 3 than Group 4 and Group 5. During the measurements, bursting sites were in the proximal and distal parts (clamp application sites) of the anastomosis.

## Discussion

Due to their high morbidity and mortality, anastomotic leakage and disruption are still serious problems in colonic operations that account for a considerable part of gastrointestinal surgery (1). In this respect, it is important to pay attention to the factors that effect anastomotic healing. These factors consist of local factors like adequate blood supply, healthy intestinal tissues, adequate bowel preparation and antibiotic therapy, surgical technique, suture materials, fecal contamination around the anastomosis and systemic factors like nutrition, shock, septicemia, acidosis, hypovolemia, severe anemia, miscellaneous medical treatments, vitamin and mineral deficiencies, age, hepatic diseases and hepatic deficiency (1,10).

In colonic surgery, various types of clamps are used in order to prevent abdominal contamination with bowel contents and bleeding from the bowel wall as well. Both mechanical trauma and ischaemia are caused in the bowel wall because of the use of clamps during anastomosis, the period of clamping, and the pressure applied to the bowel by clamps. The mucosal epithelial lesions observed after even a short period of ischaemia can be due to the damage caused by free oxygen radicals produced after reperfusion. Sadahiro *et al.* (11) reported that conventional clamps apply pressure to the center of bowel 7 times more than the bowel edges. Therefore, Ekci *et al.* (12) advised not to apply strong pressure while using intestinal clamps. Ischemia as a result of microcirculation and perfusion disorders in the tissue cause reperfusion injury and consequent wound healing problems both systemically and in the intestinal tissue. Oxygen pressure around the anastomosed segment and

Table 4. — **Bursting pressure levels**

	Bursting Pressure (mmHg) Mean $\pm$ Std.Deviation	Group 2	Group 3	Group 4	Group 5
Group 2	164 $\pm$ 11		ns	ns	ns
Group 3	132 $\pm$ 60	ns		p < 0.05	p < 0.001
Group 4	173 $\pm$ 25	ns	p < 0.05		ns
Group 5	192 $\pm$ 15	ns	p < 0.001	ns	

p < 0.05 : significant ; ns : nonsignificant.

perfusion are very important. Ischemic wound is known to heal badly and to get infected easily. That is why in surgery it is very important to decrease or prevent ischemia due to either anastomosis or clamping (1,2).

Intestinal anastomotic healing is basically according to the wound healing principles that consist of hemostasis, inflammation, proliferation and remodelling phases. With the early phase of wound healing, angiogenesis that means forming of new blood vessels from previously formed vessels starts and that has an important role in wound healing. Angiogenesis, an important phase of wound healing is inevitable in anastomotic healing and one of its most important stimulator is VEGF. Angiogenesis growth factors such as Fibroblast growth factor (FGF), VEGF, Transforming growth factor- $\beta$  (TGF- $\beta$ ), are orientated by angiogenesis stimulators like angiogenic enzymes, endothelial-specific receptors and adhesion molecules (13). VEGF, an effective endothelial mitogen, has been shown to supply vascular permeability regulation that is important at the beginning of angiogenesis (14,15). VEGF formation is induced by some growth factors and cytokines (like Platelet-derived growth factor, Epidermal growth factor, Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), TGF- $\beta$  and interleukin-1). Also amount of tissue oxygen affects VEGF. Exposure to hypoxia induces VEGF expression (13). In this study, lower levels of VEGF in Group 1 can be explained as less exposure to hypoxia than the other groups and less induction of VEGF consequently. Therefore, higher VEGF levels in Group 3 can be attributed to exposure to hypoxia and since no treatment was given to decrease ischemia, hypoxic stimulus stands still. High VEGF levels were established in the proliferative phase of wound healing (16).

As a result of reduced blood flow in the bowel related to the duration of anastomosis and squeeze power of the clamp, ischemia occurs. After the anastomosis, when the blood flow is obtained back by opening the clamps, a situation called reperfusion injury that can give more harm than ischemia, occurs (4). A popular theory that can explain mucosal and epithelial lesions shown after a very short time of reduction in blood flow of the intestine, is the occurrence of radicals as a result of reperfusion (4). As products of various reactions, oxygen radicals interact with cellular components like proteins, membranes and nucleic acids and give damage extending from cellular injury and increase in permeability to cellular

lysis (17). Affected organs included are ; heart, kidney, brain, liver, pancreas, muscles and gastrointestinal tract (4). Besides the three antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase that protect tissues from free oxygen radicals injury (15), vitamin A, beta carotene, vitamin E, vitamin C, glutathion and trace elements like copper (Cu), zinc (Zn), iron (Fe), manganese (Mn) and selenium (Se) are included in the antioxidant system (18). When the cells are exposed to the agents that produce free radicals, antioxidant enzyme levels increase. Trace elements that are included in some enzymes take part as regulators (17). Mucosal villus cells have more xanthine dehydrogenase concentration than the other tissues. As a result of this, they are the most sensitive cells to intestinal mucosal ischemic injury (18).

Various biological substances released during reperfusion injury have detrimental effects on wounds and anastomosis healing. Inhibition of collagen matrix contraction by xanthine oxidase derived reactive oxygen radicals demolishes wound healing. Additionally, by decreasing collagen production and increasing collagen destruction, cytokines and TNF- $\alpha$  diminish healing. These mediators released during reperfusion injury can inhibit wound and anastomosis healing (20).

Copper (Cu) and zinc (Zn) are mostly found trace elements in human body and by taking part in the configuration of some metalloproteinases, they participate in oxidation-reduction reactions. They are the integral components of many enzymes that have very important role in continuity of human and animal lives. Cytosolic superoxide dismutase (Cu-Zn superoxide dismutase) that has a considerable part in protection against superoxide anion is an enzyme containing iron and zinc. Lysyl oxidase also is an enzyme containing Cu, and takes part in the synthesis of cross bindings that are important in the maturation of collagen and elastin. Zinc (Zn) is a necessary trace element in growth and maturation of some organisms and in wound healing. Some enzymes containing zinc are shown that they need other elements for optimal activity. For instance, Cu is necessary for cytoplasmic superoxide dismutase activity. It has importance in stabilization and protection of plasmic and intracellular membranes, in biosynthesis and integrity of connective tissue. Therefore, it is particularly important to replace Zn after operations (21).

Papaverine, a myorelaxant and vasodilator, and pentoxifylline (PTX), a hemorrheologic agent, are used for various diseases associated with microcirculation disorders (13). Papaverine is an alkaloid from benzyloisoquinoline group derivated from opium or prepared synthetically. It causes myorelaxation in coronary, systemic, peripheral and pulmonary arteries particularly, and in smooth muscles of large vessels, gastrointestinal tract, biliary tract, uretery, bronchus and the ciliary muscle of the eye. Papaverine is recommended as a major component of the medical therapy for mesenteric ischemia. There are reports showing that papaverine causes degeneration in smooth muscles and endothelial cells of rat cerebral artery (21). In a laboratory study of Rubens *et al.*, papaverine was shown to be toxic to smooth muscle cells and this may show that some of its vasodilatory effect can be related with the death of these cells (22). Papaverine has paradoxal effects like vasoconstriction in microvascular plane (23) and secondary embolic or ischemic effects associated with precipitate formation (2).

Pentoxifylline is a methylxanthine derivative with strong hemorrheologic effect (1,3). It increases membrane deformability via increasing ATP level in erythrocyte membrane by inhibition of phosphodiesterase. Increase in membrane deformability causes erythrocyte flexibility, and this causes decrease in blood viscosity and increase in blood fluidity. PTX inhibits microvascular contraction, blocks erythrocyte and platelet aggregation, increases fibrinolysis, decreases plasma fibrinogen amounts and increases leukocyte deformability, decreases adhesion, limits leukocyte hyperreactivity by decreasing superoxide release and neutrophil adhesion (3).

PTX was shown to have protective effect against ischemia-reperfusion injury (IRI) of the intestine by inhibition of xanthine oxidase during the ischemic phase (24). Intestinal mucosa is hypersensitive to IRI. PTX treatment puts histologic proofs of IRI and biochemical marks of lipid peroxidation injury of the intestine in order (24). PTX increases blood flow in microcirculation and enhances tissue oxygenation. This hyperemic effect may cause increased neutrophil flow to anastomotic field in reperfusion period. But, Savas *et al.* (25) showed that PTX also prevented excessive flow, decreased blood flow to normal level during reperfusion and decreased intestinal IRI (26). In this study, finding a significantly higher Zn level (having an important role in the antioxidant system) in Group 4 having PTX treatment, can be regarded as a factor that supplies the antioxidant effect of PTX. Also in some experimental studies, it was shown that PTX inhibited tumor angiogenesis and this can be attributed to the inhibition of vascular endothelial growth factor, which is an important factor in angiogenesis, by PTX (27). In this study, significantly lower VEGF results in Group 4 than in Group 3, can be related to this inhibitor effect of PTX on VEGF.

As a result, surgeons should pay attention to expose tissues to ischemia as minimally as possible and replace

trace elements like Cu and Zn that are important in IRI appropriately. No encouraging effect of papaverine and pentoxifylline on the healing of anastomosis and perfusion of clamp lines has been defined. Although PTX can be respected in IRI treatment for its antioxidant characteristics, inhibition of VEGF can be considered as a disadvantage in wound healing.

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