

Sustained Local Application of Low-Dose Epidermal Growth Factor on Steroid-Inhibited Colonic Wound Healing

By A. Ebru Sakallioğlu, Aydin Yagmurlu, Huseyin Dindar, Nesrin Hasirci, Nurten Renda, and M. Salih Deveci
Ankara, Turkey

Background/Purpose: The effects of locally administered low-dose epidermal growth factor in a steroid-inhibited wound healing were investigated in a rat model.

Methods: Long-acting release of epidermal growth factor was enabled using microspheres embedded in gelatin sponge. Study groups consisted of 60 rats with 10 in each: colonic anastomosis only (C), plus pure gelatin sponge (CG), plus epidermal growth factor loaded sponge (CE), colonic anastomosis and steroid (S), plus gelatin sponge (SG), and plus epidermal growth factor-loaded gelatin sponge (SE) groups. Bursting pressure and wound hydroxy-proline content were measured. Bursting sites were recorded. Collagen deposits, inflammation, and foreign body reactions were evaluated.

Results: Bursting pressure and hydroxy-proline contents were found lowest in the S and highest in the CE groups ($P < .01$). There was almost no difference between C and SE groups. Bursts were encountered in peri-anastomotic normal

colon sites in the nonsteroid-treated C, CG, and CE groups. They were noted overwhelmingly at the anastomosis in steroid-inhibited S, SG, and SE groups. Histopathology results showed a standstill at the inflammatory phase of healing in S and SG groups. The best healing was observed in the CE group. Degree of collagen accumulation was well correlated with bursting pressure and hydroxy-proline content data with a negligible foreign body reaction to gelatin sponge.

Conclusions: Continuous local epidermal growth factor administration by microspheres in gelatin increases wound collagen and further enhances healing in colonic anastomoses even with steroid inhibition.
J Pediatr Surg 39:591-595. © 2004 Elsevier Inc. All rights reserved.

INDEX WORDS: Wound healing, colonic anastomosis, gelatin, epidermal growth factor.

LEAKAGE AFTER COLONIC anastomosis is more frequent than small bowel anastomosis and can lead to high morbidity and mortality rates. Anastomotic dehiscence may result in up to 30% mortality rate.¹

Factors reported to effect healing and integrity of the intestinal anastomosis are blood supply, anastomotic technique and meticulous procedure, colonic bacteria, inflammation, age, nutritional status, associated disease, and drugs.^{2,3}

Growth factors are small polypeptides that bind to specific cell receptors to play a significant role in the stages of wound healing, including the synthesis, deposition, and maturation of collagen. A wound cytokine, epidermal growth factor (EGF), has a mitogenic effect on wound healing because it is produced and secreted by local inflammatory cells.⁴ It activates fibroblast proliferation, consequently enhancing new collagen synthesis.⁵ EGF receptors are present in many tissues with their acknowledged effect on wound healing and growth.⁶ A low-level EGF gene expression has been shown to correlate with a significant increase in EGF receptor gene expression after colonic anastomosis.⁵

It is postulated that local administration of exogenous EGF in the anastomotic healing process will benefit wound healing, especially when it is impaired, because EGF receptors are present in newly performed colonic anastomosis. This study was intended to surface the

effects of local EGF release from biocompatible and biodegradable gelatin microspheres^{7,8} embedded in porous sponge used in normal and steroid-inhibited colonic wound healing.

MATERIALS AND METHODS

Sixty female (180 to 210 g) Sprague-Dawley rats, housed in a controlled temperature, humidity, and 12-hour light-dark cycle were fed with food and water ad libitum. After the approval of the research protocol by the Institutional Ethics Committee (12.01.2001-1033), rats were assigned randomly to one of 6 groups containing 10 animals in each. All animals underwent colonic anastomosis. The first 3 groups of rats received daily 0.1-mg/kg dexamethasone-sodium phosphate intramuscularly for 14 days preoperatively: just anastomosis (S, n = 10), pure sponge (SG, n = 10), and EGF-loaded sponge (SE, n = 10) groups. Next, 3 groups were accepted as control; animals received daily

From the Department of Pediatric Surgery, Baskent University, Faculty of Medicine; the Department of Pediatric Surgery, the Ankara University, School of Medicine; the Department of Chemistry, Middle East Technical University, Faculty of Art and Sciences; the Department of Biochemistry, Hacettepe University, Faculty of Medicine; and the Department of Pathology, Gulhane Military Medical Academy, School of Medicine, Ankara, Turkey.

Address reprint requests to A. Ebru Sakallioğlu, Baskent University, School of Medicine, Department of Pediatric Surgery, Fevzi Cakmak Cad. 10.Sok No:45, Bahçelievler, Ankara, Turkey.

© 2004 Elsevier Inc. All rights reserved.
0022-3468/04/3904-0017\$30.00/0
doi:10.1016/j.jpedsurg.2003.12.017

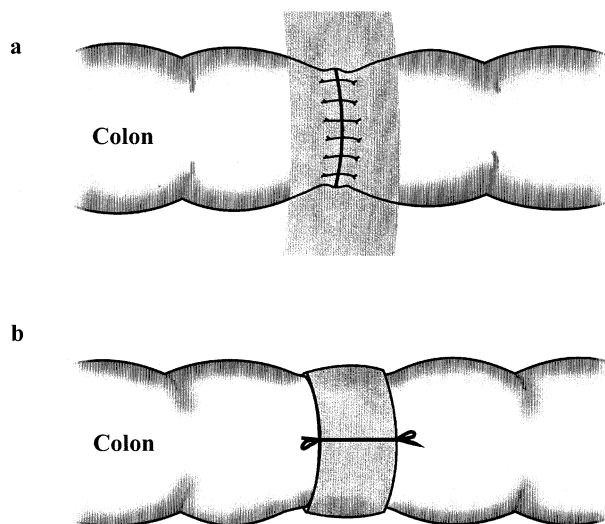


Fig 1. Schematic scene of the surgical procedure used for gelatine-applied groups.

intramuscular injections of 0.5 mL isotonic saline instead of steroids: just anastomosis (C, n = 10), pure sponge (CG, n = 10), EGF-loaded sponge (CE, n = 10) groups.

Rats were anesthetized (Ketamine HCl 50 mg/kg intramuscularly) and underwent midline laparotomy after cephalosporin sodium (100 mg/kg) administration. A segment measuring 1 cm of the descending colon was resected at a level 3 cm proximal to the pelvic brim. Anastomosis was performed with 10 interrupted 7-0 atraumatic polypropylene sutures (Ethicon, Edinburgh, UK) placed in an inverted manner in all groups. Pure or EGF-loaded sponge (4 × 0.5 × 0.2 cm) was placed onto the serosal surface of the anastomosis with 2 stitches of 5-0 atraumatic chromic-catgut in CG, CE, SG, and SE groups (Fig 1A & B). The abdominal wall was closed with 2 layers of continuous 3-0 polyglactin (Ethicon), and animals were returned to their cages and given food and water ad libitum. After 7 days of saline or dexamethasone injections, a second-look laparotomy was performed. Segments of colon of 2.5 cm with centrally situated anastomosis were prepared from each animal. Bursting pressures (BP) were measured with the infusion of saline in a speed of 49.9 mL/h to this isolated segment through a plastic catheter that was connected to a pressure transducer. Bursting sites were determined as described by Koruda and Rolandelli.⁹ Two-centimeter-length tissue samples including anastomosis line were taken and incised longitudinally. A piece of the specimen was fixed in 10% formalin, and the rest was snap-frozen in liquid nitrogen and stored at -80°C for determination of hydroxy-proline (HP) content. HP content was measured by spectrophotometrical method described by Bergeman and Loxly.¹⁰

For examination of acute inflammation and foreign body reaction, histologic specimens were investigated by light microscopy after being stained with H&E, whereas collagen distribution was evaluated with the same manner using trichrom stain after saline wash.

Preparation of Sponge

Aqueous gelatin solution (1.5 g in 45 mL) was stirred at 2,000 rpm for 15 minutes. And 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was added as cross-linking agent to stabilize the spongy structures while mixing for another 5 minutes. The spongy solution was put into 0.5-cm-high glass moulds treated with silicon to prevent adhesion and freeze dried. Sponges, containing EGF-loaded microspheres, were prepared in the same way with addition of microspheres into the spongy solution just before molding.

EGF containing microspheres were produced by water-oil inverse-emulsion polymerization technique by using EGF (200 µg) containing aqueous gelatin solution (1 g/15 mL) in mineral oil (35 mL). Microspheres were hardened with glutaraldehyde solution (2 mL of 0.5%), cleaned by acetone and then dried. The amount of EGF in gelatine sponge was calculated as 1.05 µg/cm², thus, enabling EGF administration to the anastomotic line throughout 4 days in a calculated mean dose of 1 µg/kg/d.

Data were presented as mean ± SEM. A *P* value of less than .05 was considered as statistically significant. A nonparametric 2-way analysis of variance (ANOVA) test was used to test the differences.

RESULTS

All anastomoses were intact with no apparent obstruction or stenosis. Bursting occurred at the anastomosis in 1 of 10 animals (10%) in CG, 2 of 10 (20%) in CE, and 3 of 10 (30%) in C for those groups without steroid inhibition. For the steroid-applied S group, the bursting sites were observed on anastomotic line in 10 animals (100%) implying retarded healing. Application of sponge or EGF containing sponge resulted in anastomotic bursting in 8 of 10 animals (80%) and 7 of 10 (70%) for SG and SE groups, respectively (Table 1).

The BP of CE was found as 219 ± 12.29 mm Hg and significantly higher than all the other groups (*P* < .01). For steroid-applied S and SG groups, BP was significantly reduced as 14.14 ± 2.58 mm Hg and 18.62 ± 1.59 mm Hg, respectively (*P* < .01). But it was increased up to 181.71 ± 14.58 mm Hg in the EGF-applied steroid group SE, and found to be very similar to the C (180.20 ± 8.7 mm Hg) and CG (177.44 ± 7.27 mm Hg) groups (Table 1).

The highest concentration of tissue HP was found in the CE group (4.52 ± 0.20 µg/mg tissue), parallel to the bursting pressure measurements (*P* < .01). Minimal HP was encountered in S and SG groups (*P* < .01) and quantified as 2.59 ± 0.21, 2.66 ± 0.19 µg/mg tissue, respectively. HP value reached to 3.01 ± 0.18 µg/mg tissue for SE group, which is quite close to C and CG groups' results (3.47 ± 0.19 and 3.31 ± 0.19,

Table 1. The Bursting Sites, Bursting Pressures, and Tissue Hydroxyproline Levels

	C	CG	CE	S	SG	SE
Anastomotic bursting site (%)	30	10	20	100	80	70
Bursting pressure (mm Hg)	180.2 ± 9	177.4 ± 7	219 ± 12*	14 ± 3	18.6 ± 2	181.7 ± 5†
Hydroxyproline content (µg/mg tissue)	3.47 ± 0.19	3.31 ± 0.19	4.52 ± 0.2*	2.59 ± 0.21	2.66 ± 0.19	3.01 ± 0.18†

*Significantly different than all other groups (*P* < .05).

†Significantly higher than S (*P* < .05) and SG but not different than C and CG.

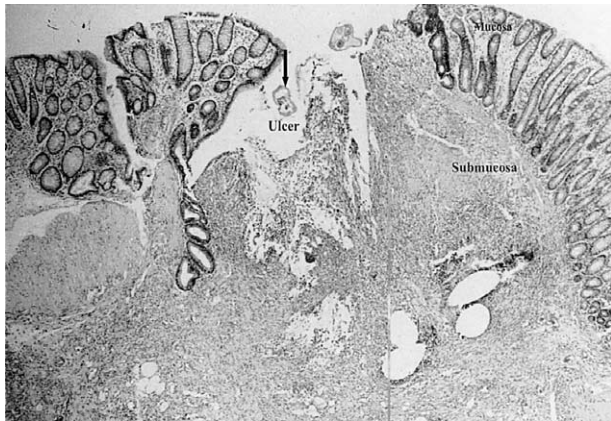


Fig 2. Incomplete epithelization and a huge ulcer in mucosa in S group (H&E original magnification $\times 100$).

respectively). These measurements correlated with BP (Table 1).

Histopathologic examination found a delayed reaction at the inflammatory phase of healing in S and SG groups with high portion of polymorphonuclear (PMN) leukocytes and incomplete epithelization with ulcers in the mucosa (Fig 2). The best healing process was observed in the CE group with a good amount of organized collagen accumulation (Fig 3). Tissues including sponge (EGF loaded or not) showed scarce biomaterial remnants with crossings of collagen fibers (Fig 4).

Minimal foreign body reaction next to the sponge remnants was observed in 2 animals of the CG group, in 1 animal in CE, of SG and 1 in SE group. Neither remnants of chromic catgut nor a noteworthy foreign body reaction around polypropylene remnants in the samples of the anastomotic area was observed.

DISCUSSION

Attempts to support production and maturation of new collagen are beneficial for prevention of anastomotic leakage. The anastomotic healing is effected by the degree of primary inflammatory response; the rate of mucosal reepithelization; the amount, strength, and maturation rate of new collagen; and collagenolysis in the initial 3 days of the postanastomotic period.^{11,12} The strength of an anastomosis is based on the collagen fibers and their maturation at the submucosa.¹¹

Data from the current experiments show that the local and continuous EGF administration resulted in greater anastomotic strength. The colonic wall of the EGF-applied group (CE) resisted greater intraluminal pressures than all other animals. Bursting pressure of the EGF-applied steroid group (SE) was similar to the non-steroid-applied control rats (C). Data from the current study also indicate that hydroxyproline correlates significantly with the BP.

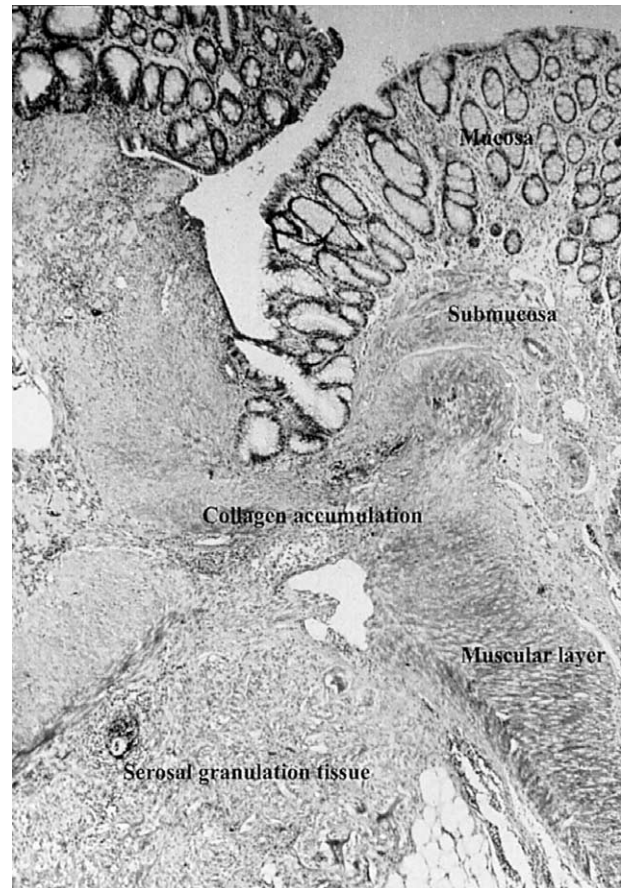


Fig 3. A good amount of organized collagen accumulation in CE group (Trichrom-Mason, original magnification $\times 200$).

EGF, platelet-derived growth factor BB, and transforming growth factor- β are considered as active cytokines in colonic wound healing. EGF and EGF receptor gene expression in colon indicate that the number of EGF receptors increases significantly with no changes in EGF gene expression levels.¹³ Another endogenous cy-

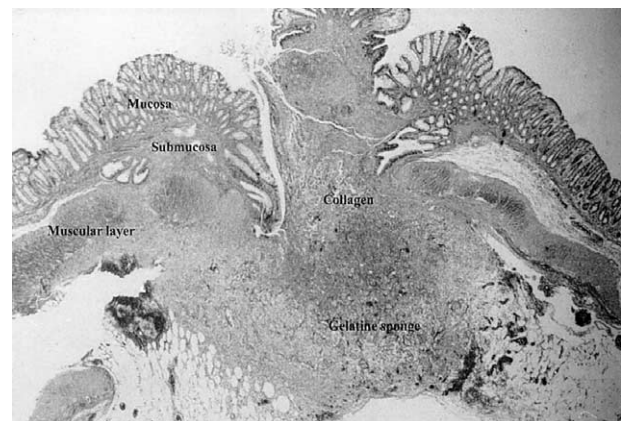


Fig 4. Little amount of gelatine sponge remnants observed in CE group (Trichrom-Mason, original magnification $\times 100$).

tokine, transforming growth factor- α , binds to these receptors.⁵

Increase of EGF receptors at colonic anastomotic sites during the wound healing process could be a possible explanation about the beneficial effects of locally administered EGF in the current study. This hypothesis can also be supported with the study by Kingsnorth et al, which suggested the enhancement of colonic anastomosis healing with the administration of intraperitoneal EGF.¹⁴

Synthesis of collagen is maximized in 5 to 7 days by proliferation of collagen-producing local fibroblasts.^{6,15} Therefore, EGF was chosen to be released during the initial postoperative 4 days for enhancing the fibroblastic proliferation during the healing process in the current study. Inadequate blood supply, bacterial count of colon, inflammation, technical errors, age, nutritional status, associated diseases, and drugs such as chemotherapeutics, octreotide, suramine, irradiation, and dexamethasone impair the wound healing process.^{2,3,12,16-18} In the current study, the most significant effect of locally administered EGF in gelatin microspheres was shown in steroid-administered groups. Local administration of EGF by controlled release in the SE group established a 1,185% higher BP than the steroid-administered S group.

Dexamethasone is known to decrease the inflammatory cell functions including any cytokine secretion.¹⁹ High levels of BP in the SE group might be the result of the cytokine provided exogenously, where steroids restricted endogenous secretion from inflammatory cells.

The effects of a number of chemical, physical, and nutritional elements have been investigated on the normal or inhibited wound healing process of colonic anastomosis such as arginine, short chain amino acids, vitamin A, zinc, He:Ne laser, erythropoetin, and hyperbaric oxygen.²⁰⁻²⁶ Increase of bursting pressure values to that of controls were 32% with short chain fatty acids,²⁰ 24% with arginine,²¹ 37% with erythropoetin.²² Intraperitoneally administered EGF increased BP by 12%.¹⁴ The current study of local EGF administration indicated a 22% higher BP than the controls in nonsteroid-inhibited wound healing.

Systemic application of vitamin A in a subtoxic dose

increased the tensile strength 56% higher than the controls, and prostaglandin E1 to 68% in a previous steroid inhibited wound healing model of colonic anastomosis with some reported side effects caused by PGE1.²³ We did not examine any side effects throughout the study. Bursting occurred at extra-anastomotic sites in 30% of animals of the SE group indicating that the healing was stronger than S and SG groups.

In the current study, the amount of EGF in the sponge structures placed onto the anastomosis line was 1.05 $\mu\text{g}/\text{cm}^2$. This amount of the cytokine is proposed to be administered to the anastomotic line throughout 4 days in a dose of 1 $\mu\text{g}/\text{kg}/\text{d}$. When compared with 150 to 200 $\mu\text{g}/\text{kg}/\text{d}$ systemic dose of EGF used in previous studies,^{24,25} there is a significant benefit for cost effectiveness by sustained release of EGF.

These cost-effective results of the study were supplied by the help of gelatin microspheres loaded onto biocompatible and biodegradable sponge. Those microspheres were used as vehicles, which released EGF continuously during the postoperative period.

Presence of receptors previously identified on colorectal cancer cells hinders administration of local EGF in malignancy.¹ In an experimental rat tumor model, application of collagen, with or without growth factors, increased the number of tumor-developing animals and peri-anastomotic tumor growth. Collagen, and consequently gelatin, therefore, must be considered only as a vehicle for nonmalignant entities of impaired wound healing until further studies enlighten the issue. R0-resected patients could be an exception, although there seems to be no reason that they would suffer or take other nonwanted complications caused by gelatin sponges or EGF.

The current study shows that local, continuous, and low-dose EGF administration provided by gelatin microspheres increases collagen and enhances the healing process even in steroid-inhibited colonic anastomosis cost effectively. A possible mechanism is that exogenous EGF administration to the anastomotic line may have bound to the EGF receptors and enhanced the proliferation of collagen-producing fibroblasts. Further studies are required to clarify the mechanism.

REFERENCES

1. Reinbach D, McGregor JR, Dahill SW, et al: Collagen promotes perianastomotic tumor growth in an experimental animal model. *J Surg Oncol* 60:112-115, 1995
2. Buckmire MA, Parquet G, Greenway S, et al: Temporal expression of TGF- β 1, EGF and PDGF-BB in a model of colonic wound healing. *J Surg Res* 80:52-57, 1998
3. Van Der Ham AC, Kort WJ, Weijma IM, et al: Transient protection of incomplete colonic anastomosis with fibrin sealant: An experimental study in the rat. *J Surg Res* 55:256-260, 1996
4. Dudrick PS, Copeland EM, Bland KI, et al: Divergent regulation of fuel utilization in human fibroblasts by epidermal growth factor. *J Surg Res* 54:305-310, 1993
5. Brasken P, Renvall S, Sandberg M: Expression of epidermal growth factor receptor genes in healing of colonic anastomosis in rats. *Eur J Surg* 157:607-611, 1991
6. Franzen L, Ghassemifar R, Schultz G: Specific binding of EGF in connective tissue repair. *Eur J Cell Biol* 60:346-350, 1993
7. Pluta J, Haznar D: Resorbed polymeric sponge used as a

carrier the therapeutic substance. Part 1. *Polym Med* 31:18-25, 2001

8. Ulubayram K, Çakar AN, Korkusuz P, et al: EGF containing gelatine-based wound dressings. *Biomaterials* 22:1345-1356, 2001

9. Koruda MJ, Rolandelli R: Current research review, experimental studies on the healing of colonic anastomoses. *J Surg Res* 48:504-515, 1990

10. Bergeman I, Loxly R: Two improved and simplified methods for the spectrophotometric determination of hydroxy-proline. *Ann Chem* 35:1961-1965, 1963

11. Brasken P, Lehto M, Renvall S: Changes in the connective tissue composition of the submucosal layer of colonic anastomosis. *Acta Chir Scand* 155:413-419, 1989

12. Schorck TR, Deveney CW, Dunphy JE: Factors contributing to leakage of colonic anastomoses. *Ann Surg* 177:513-518, 1973

13. Cohen IK, Diegelman RF, Yager DR, et al: Wound care and wound healing, in Schwartz SI, Shires TG, Spencer FC, et al (eds): *Principles of Surgery*. McGraw-Hill, 1999, pp 263-296

14. Wovles R, Nash JRG: Epidermal growth factor increases tensile strength in intestinal wounds in pigs. *Br J Surg* 77:409-412, 1990

15. Cohen S, Orth DN: Isolation of mouse submaxillary gland protein accelerating incisor eruption and eye-lid opening in the new born animal. *J Biol Chem* 237:1555, 1962

16. Hedricks JM, Hubens G, Wuyts FL, et al: Experimental study of intraperitoneal suramin on the healing of colonic anastomosis. *Br J Surg* 86:1171-1175, 1999

17. Kuzu MA, Kuzu I, Köksoy C, et al: Histological evaluation of colonic anastomotic healing in the rat following preoperative 5-flour-

ouracil, fractioned irradiation and combined treatment. *Int J Colorectal Dis* 14:235-240, 1999

18. Türkçapar AG, Demirel S, Sengül N, et al: The adverse effects of octreotide on the healing of colonic anastomosis. *Surg Today* 28:279-284, 1998

19. Harvey W, Grahame R, Panayi GS: Effects of steroid hormones on human fibroblasts. *Ann Rheum Dis* 33:437-441, 1974

20. Saclarides TJ, Rohrer DA, Bhattacharya AK, et al: Effects of intraoperative radiation on the tensile strength of intestinal anastomosis. *Dis Colon Rectum* 35:151-157, 1992

21. Shashidharan M, Lin KM, Ternent CA, et al: Influence of arginine dietary supplementation on healing colonic anastomosis in the rat. *Dis Col Rectum* 42:1613-1617, 1999

22. Fatouros MS, Vekinis G, Bourantas KL, et al: Influence of growth factors erythropoietin and granulocyte macrophage colony stimulating factor on mechanical strength and healing of colonic anastomoses in rats. *Eur J Surg* 165:986-992, 1999

23. Cali RL, Smyrk TC, Blatchford GJ, et al: Effects of prostaglandin and steroid on healing colonic anastomoses. *Dis Colon Rectum* 36:1148-1151, 1993

24. Kissmeyer-Nielsen, Vinter-Jensen L, Smerup M: Effects of long term epidermal growth factor treatment on the normal rat colon. *Gut* 38:582-586, 1996

25. Luck MS, Bass P: Effects of epidermal growth factor on experimental colitis in the rat. *J Pharmacol Exp Ther* 264:984-990, 1992

26. Hamzaoğlu İ, Karahasanoğlu T, Aydın S, et al: The effects of hyperbaric oxygen on normal and ischemic colon. *Am J Surg* 176:458-461, 1998