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Original Research

VEGF-A and FGF gene therapy accelerate healing of ischemic colonic anastomoses (experimental study)

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ABSTRACT

Background: Reducing ischemic damage is one of the goals of surgery. The aim of this study was to apply human VEGF-A and FGF-2 DNA-mediated gene therapy in order to identify their effects in the healing of ischemic colon anastomoses and eliminating the negative effects of ischemia.
Methods: Forty male Wistar albino rats weighing 250–280 g were divided into five equal groups ($n = 8$) as follows: group 1: control, ischemic left colonic anastomosis; group; 2: ischemic left colonic anastomosis with control plasmid delivery; group 3: ischemic left colonic anastomosis with VEGF plasmid delivery; group 4: ischemic left colonic anastomosis with FGF plasmid delivery; group 5: ischemic left colonic anastomosis with VEGF and FGF plasmid delivery. All rats were sacrificed on the 4th postoperative day. Anastomosis burst pressures were measured for mechanical examination of anastomosis. Tissue hydroxyprolin, VEGF and FGF levels were determined as biochemical parameters. Necrosis, epithelisation, inflammatory processes, fibroblastic activity, collagen deposition and neovascularisation at the anastomic site were studied.
Results: VEGF, FGF and combined therapy significantly accelerated many of the histological parameters of healing, including fibroblast activation, collagen deposition, and angiogenesis, and augmented the levels of hydroxyproline and bursting pressure.
Conclusions: This is the first study to use gene therapy with growth factors for the healing of ischemic colonic anastomosis. This therapy can be effectively used in increasing ischemic anastomosis wound healing.

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1. Introduction

Ischemia is one of the most important contributing causes of dehiscence of intestinal anastomoses. After gastrointestinal surgery, many instances of leakage during clinical situations exhibit hypoperfusion of anastomoses. If the degree of hypoxia is enough to interfere with tissue viability, tissues can necrose and lead to delayed wound healing. Preventing or reducing the ischemic damage is one of the goals of surgery.[1,2]

Growth factors (GF) are the engines or modulating factors that drive wound healing. Through their induction of chemotaxis and cell proliferation and by stimulating cells to upregulate protein production, they determine extracellular matrix synthesis, matrix turnover, and cell death.[3,4] In the gastrointestinal system, the repair process is initiated after anastomoses by the release of various growth factors.[5] The fibroblast growth factor (FGF) family has a broad mitogenic spectrum, and members of this family stimulate proliferation of various cells of mesodermal, ectodermal, and endodermal origin.[5] Vascular endothelial growth factor (VEGF), especially VEGF-A, has been identified as a major regulator of vasculogenesis and angiogenesis during development, indicating that it may also be involved in the regulation of angiogenesis during wound healing.[6]

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Gene therapy is a new and emerging technology that employs the process of manipulating genes and involves the insertion of a desired gene (the transgene) into the recipient's cells.[7] Plasmids are molecules of DNA that are found in bacteria but are distinct from the bacterial chromosome. Plasmid DNA has a number of advantages over viral vectors as a vector for non-viral gene therapy.[8] The direct injection of naked plasmid DNA into the target tissue enables transgene expression.[9] The simplicity of injecting naked plasmid DNA into tissue with a syringe is an attractive way to conduct gene therapy research. The addition of growth factors into the target tissue in order to enhance wound healing is a new approach. In this study, human VEGF-A and bFGF-2 genes were separately incorporated into two recombinant plasmids in order to identify their effects on the healing of ischemic colon anastomosis and eliminate the negative effects of ischemia.

2. Materials and methods

This study was completed in the Department of Genetics at The Institute of Experimental Medicine, Istanbul University. The study protocol was approved by the Institutional Animal Care and Use Committee at Istanbul University. Forty male Wistar albino rats weighing 250–280 g were divided into five equal groups ($n = 8$). The animals were housed at 21 °C and were given tap water and standard rat food.

Group 1: (Control) Ischemic left colonic anastomosis
Group 2: (Control) Ischemic left colonic anastomosis + plasmid delivery
Group 3: Ischemic left colonic anastomosis + VEGF-A plasmid delivery
Group 4: Ischemic left colonic anastomosis + FGF plasmid delivery
Group 5: Ischemic left colonic anastomosis + VEGF-A and FGF plasmid delivery

All of the rats were sacrificed on postoperative day 4. Anastomosis burst pressures were measured for mechanical examination of anastomosis. Tissue hydroxyproline, VEGF and FGF levels were examined for biochemical parameters. Necrosis, epithelisation, inflammatory processes, fibroblastic activity, collagen deposition and neovascularisation at the anastomic site were studied using a light microscope.

2.1. Surgical procedure

After one night of fasting, the animals were anaesthetised by an intramuscular injection of ketamine hydrochloride (50–100 mg per kg of body weight). Abdominal access was achieved through a midline incision 4 cm long, and the left colon was diverted 3 cm proximal to the peritoneal reflection. In order to establish ischemic colon anastomosis, vessels in the mesocolon between 2 cm proximal and 2 cm distal to the anastomosis line were ligated. After the faecal contents had been removed, a standardised end-to-end anastomosis was made from eight interrupted, inverted sutures of 6/0 polypropylene. After the surgical procedure, group 2 received local injection of 2 μg plasmid in 0.5 mL 0.9% NaCl into the tissues surrounding the anastomotic area. Groups 3 and 4 were given VEGF-A plasmid or FGF plasmid with physiological serum (0.5 ml 0.9% NaCl, 1 μg VEGF-A, 1 μg FGF) and the last group (group 5) was given VEGF-A and FGF plasmid (0.5 ml 0.9% NaCl + 1 μg VEGF-A + 1 μg FGF), all locally into the tissues surrounding the anastomotosis. Once surgical bleeding was under control, 2 cc of 0.9% NaCl was injected intraperitoneally and the abdomen was closed with 3/0 continuous silk sutures. Water was given after 12 h and food was given 24 h after the surgery.

2.2. Plasmid constructions and injections

VEGF-A and FGF-2 expression plasmids were constructed separately by ligating full-length human VEGF-A (Gene ID: 7422) and FGF-2 (Gene ID: RR) cDNAs into pcDNA4 (Invitrogen). Briefly, total RNA was isolated from cultured cells using the total RNA purification kit according to the manufacturer's recommendations (Qiagen). After reverse transcription, PCR was amplified with the VEGF-A forward oligo 5′-GAATTCGCCGACATGACGGACAGACAGACAGACACCGCC-3′ containing an EcoRI site and the reverse oligo 5′TCTAGATCACCGCCTCGGCTTGTCACATCTGC-3′ with a modified XbaI site at the 5′ and 3′ ends. The FGF-2 forward 5′- GAATTCGCCACATGGTGGGTGTGGGGGGTGGAGATGTAGA-3′ oligo containing EcoRI and the reverse oligo 5′-TCTAGATCAGCTCTTAGCAGACATTGGAAGA-3′ with an XbaI site were created. The purified PCR fragments and pcDNA4 were double digested with EcoRI and XbaI restriction enzymes. The digested fragments were recovered from a 1% low melting agarose gel and DNA was eluted from the sliced agarose gel using a gel extraction kit (Qiagen). Fifty nanogrammes of EcoRI- and XbaI-digested and dephosphorylated vector DNA and 1 μl of PCR product were incubated with DNA ligase buffer containing T4 DNA ligase at 16 °C overnight. Three microlitres of ligation reaction and 100 μl of DH5 alpha competent *E. coli* were heat-shock transformed at 42 °C and spread on agar plates containing 100 mg/ml ampicillin.

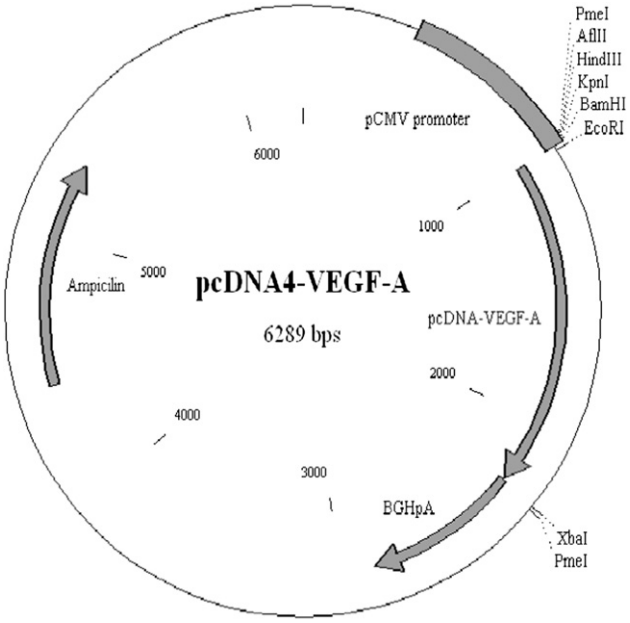

Fig. 1. VEGF-A plasmid construct.

Plates were incubated overnight at 37 °C. PCR-tested and selected positive clones were confirmed by sequencing (Refgen Co, Turkey). Plasmids were purified using an Endofree maxi Plasmid kit (Qiagen Co). A ratio of 1:1 the of two plasmid constructs, each with either human VEGF-A (1 μg) (Fig. 1) or FGF-2 (1 μg) (Fig. 2) DNA were mixed and administered onto the local anastomosis of the rat colon. In addition, an empty pcDNA4 plasmid (2 μg) was used as a control.

2.3. Measurement of colonic bursting pressure

The abdominal incision was reopened and the anastomotic sutures lines were identified. The anastomotic segment was found and bursting pressure was measured *in situ* without detaching adhesions. The bursting pressure of the anastomotic segment was measured with a mercury manometer and a constant flow pump. Briefly, a 16-gauge silastic catheter was inserted via the anal canal. The proximal section of the anastomosis was ligated with 2/0 silk and continuously infused through the catheter via a tube pump at a rate of 4 ml/min. Bursting pressure was recorded (mmHg) as the peak pressure attained before rupture of the anastomosis, which resulted in an abrupt drop in pressure.

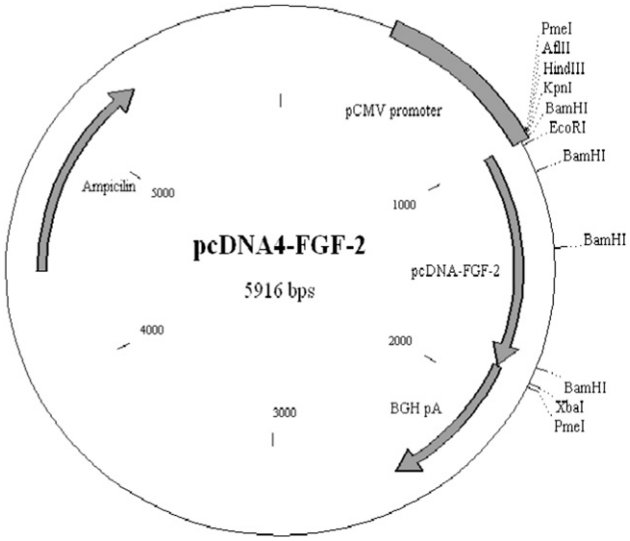

Fig. 2. FGF-2 plasmid construct.

Table 1
Bursting pressures and hydroxyproline contents in all groups. *p* value comparison between group 1 (control) and groups 2–5. All values expressed as mean ± SD.

Bursting Pressure (mmHg)					
	Group 1 (control)	Group 2 (empty plasmid delivery)	Group 3 (VEGF-A plasmid)	Group 4 (FGF plasmid)	Group 5 (VEGF-A + FGF plasmids)
Mean ± SD	59.4 ± 20	91.3 ± 22	106.3 ± 21	103.8 ± 22	126.3 ± 27
p		$p < 0.05$	$p < 0.01$	$p < 0.01$	$p < 0.01$
Hydroxyproline content (μg/mg)					
Mean ± SD	0.42 ± 0.07	0.43 ± 0.08	0.51 ± 0.09	0.8 ± 0.05	1.5 ± 0.04
p		$p > 0.05$	$p < 0.05$	$p < 0.01$	$p < 0.01$

*SD: Standard deviation.

2.4. Hydroxyproline, VEGF and FGF determination

The tissue samples were excised 0.5 cm proximal and 0.5 cm distal to the anastomosis line. All of the samples were then weighed and homogenised in physiological serum as 20% homogenates using a Potter type glass homogeniser (Heidolphy-RZR 2021, Germany). The homogenates were centrifuged at 1500 g for 15 min and the obtained supernatants were hydrolysed by adding equal amounts of hydrochloric acid for 10–18 h. Using a hydroxyproline kit (Hipronisticon, Organon, Holland) that is based on the principles of Stegeman and Stadler, the hydroxyproline amount was calculated in micrograms per milligram of wet tissue by reading the absorbance of the solution on a spectrometer at 560 nm. Total VEGF and FGF levels were measured using VEGF and FGF ELISA kits (R&D Systems, USA). The measurements were conducted according to the methods recommended by the manufacturer. The minimum detection limit for total VEGF was 31 pg/ml and the limit for FGF was 1.0 pg/ml. All of the measurements were calculated while blinded to the group identity.

2.5. Histopathological examinations

To determine histological and immunohistochemical assessments, the bursting colon was excised, cleared of surrounding mesentery and fat, and washed with saline. The anastomosis was excised together with 1 cm of the adjacent proximal and distal colon. The tissues were fixed in 10% formalin for approximately 24 h and then embedded in paraffin. Transverse sections of the embedded tissue that were 3 μm in thickness were stained with haematoxylin and eosin, and histological assessment was carried out by an experienced pathologist who was blinded to the identity of the groups. Necrosis, epithelisation, inflammatory processes, fibroblastic activity, and neovascularisation at the anastomic site were studied and scored (from 0 to 3, where 0 is none, 1 is slight, 2 is moderate and 3 is dense). Masson's trichrome staining was used to distinguish cells from the surrounding connective tissue. Three dyes were employed and solution C was used to stain collagen. Collagen deposition was scored according to the density of the colonic tissue (from 0 to 3, using the aforementioned scale).

Statistical analysis. Data are reported as mean ± SD. All statistical analyses were performed using SPSS 16.0 for Windows. The Mann–Whitney *U* test was used to compare continuous data between groups. Categorical variables are presented as counts and percentages, and the Kruskal–Wallis and paired *t*-tests were used to assess associations between discrete and group variables. A probability value of $P < 0.05$ was considered to denote statistical significance.

3. Results

3.1. Bursting pressure

The mean bursting pressure of ischemic colonic anastomoses in group 5 was the highest of all of the groups (126 mmHg) (Table 1). The bursting pressures in experimental groups 3–5 were significantly different from those in the group 2($p < 0.05$, $p < 0.01$) (Table 1).

3.2. Hydroxyproline content

We found significantly higher hydroxyproline levels in groups 3–5 compared to the control group. Hydroxyproline levels were increased in groups that had received growth factor therapy. Hydroxyproline values for all groups are given in Table 1.

3.3. VEGF and FGF determination

We found significantly higher VEGF levels in the tissue of groups 3 and 5 compared to the control group ($p < 0.01$) (Table 2). The FGF levels were also higher in groups 3–5 compared to group 1 ($p < 0.01$) (Table 2).

3.4. Histological features

There was no mortality in any of the groups. We investigated the early period of wound healing. We found higher levels of many histological parameters such as collagen deposition, fibroblast activity and angiogenesis in groups 3 and 5 compared to the control group 1 (Table 3, Fig. 3). In addition, we also found lower levels of necrosis in groups 3, 4 and 5. There were no significantly differences in histological parameters, such as epithelisation and inflammation, between any of the groups ($p > 0.05$).

4. Discussion

Necrosis following hypoxia is one of the most important causes of anastomotic leakage. Despite improvements in surgical and medical techniques, the rates of anastomotic leakage remain high; this necessitates the investigation of new agents and new methods. The success of the wound healing process depends on growth factors, cytokines, and chemokines in a complex integration of signals that coordinate cellular processes. These agents are biologically active polypeptides that act to alter the growth,

Table 2
Tissue VEGF and FGF level in all groups. *p* value comparison between group 1 (control) and groups 2–5. All values expressed as mean ± SD.

Tissue VEGF-A and FGF level (pg/ml)					
	Group 1 (control)	Group 2 (empty plasmid delivery)	Group 3 (VEGF-A plasmid)	Group 4 (FGF plasmid)	Group 5 (VEGF-A + FGF plasmids)
Mean ± SD	200.5 ± 15	258.6 ± 39	435 ± 18	280 ± 52	431 ± 14
p		$p > 0.05$	$p < 0.01$	$p > 0.05$	$p < 0.01$
Tissue FGF Level (pg/ml)					
Mean ± SD	0.91 ± 0.16	1.03 ± 0.22	1.58 ± 0.2	2.57 ± 0.19	2.73 ± 0.15
p		$p > 0.05$	$p < 0.01$	$p < 0.01$	$p < 0.01$

*SD: Standard deviation.

Table 3
Histological features that characterise the healing of ischemic anastomosis. All of the parameters were scored from 0 to 3. *p* value comparison between group 1 and groups 2–5.

	Group 1	Group 2	Group3	Group 4	Group 5
Necrosis	2.8	2.4	1.5	1.6	1.2
p		$p > 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.01$
Epithelialisation	1.4	1.3	1.4	1.9	2
p		$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Collagen deposition	1.1	1.8	2	2.4	2.3
p		$p > 0.05$	$p > 0.05$	$p < 0.01$	$p < 0.01$
Fibroblast Activity	1	2	2.5	2.8	2.8
p		$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$
Inflammation	2.4	2.9	2.6	2.8	2.5
p		$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Angiogenesis	1.3	1.6	2.5	2.6	2.6
p		$p > 0.05$	$p < 0.01$	$p < 0.01$	$p < 0.01$

differentiation and metabolism of a target cell.[10] The growth factors that play essential roles in wound healing, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF), can be referred to as connective tissue growth factors given their local function and rare systemic effects.[11,12] Little is known about the ratio of growth factor concentrations in tissue, which may be as important as the absolute concentration of individual growth factors.[3] Gene therapy in wound healing holds significant promise as a tool for understanding the role of growth factors in wound healing and as a therapeutic strategy.[13] There have been no studies in the literature that address the delivery of growth factors via plasmids for enhancement of ischemic wound healing. This study demonstrated that FGF and VEGF-A gene transfer can be used safely and successfully by local injection in ischemic colonic anastomoses. Moreover, the study showed that this therapy significantly accelerated many parameters of the healing process for ischemic colonic anastomoses. Although there is a large body of work in the literature demonstrating the effects of growth factors in animals, translation of these data into clinical practice has had limited success.[3]

Assessment of anastomic healing mainly depends on mechanical parameters, and measurement of bursting pressure is particularly informative.[14,15] Bursting pressures have been shown to remain low for the first 3–4 days after surgery.[16,17] Bursting pressure is the most commonly used method to assess anastomic healing.[18] The lowest level of bursting pressure (59 mmHg) was found in group 1 (control). The bursting pressure was approximately 2 times greater in group 5 (126 mmHg) than in the control, where this difference was statistically significant ($p < 0.01$) (Table 1). The higher bursting pressure measurements in the VEGF-treated animals corresponds with studies that have shown the opposite trend in animals treated with anti-angiogenic agents.[19] Hydroxyproline levels, which indicate collagen content, were determined. Hydroxyproline is found almost exclusively in collagen and serves as a marker of the quantity of collagen in tissue.[20] In this study, bursting pressure and hydroxyproline levels showed that therapy with growth factors augmented the healing process in ischemic colonic anastomoses in groups 3, 4 and 5 (Table 1).

Numerous growth factors are produced during the healing process. These growth factors stimulate continued wound cellular proliferation, angiogenesis, and production of extracellular matrix proteins and glycoproteins.[18] With the assistance of platelet-released VEGF and FGF, endothelial cells proliferate and angiogenesis ensues. This process is essential for the synthesis, deposition and organisation of new extracellular matrix.[10] FGF levels are increased in the acute wound and play a role in the formation of granulated tissue, re-epithelisation, and tissue remodelling.[10] A characteristic feature of FGF is its interaction with heparan sulphate proteoglycans, which stabilises FGF from thermal denaturation and proteolysis.[21,22] The VEGF family, especially VEGF-A, has been identified as a major regulator of vasculogenesis and angiogenesis during development, indicating that it might also be

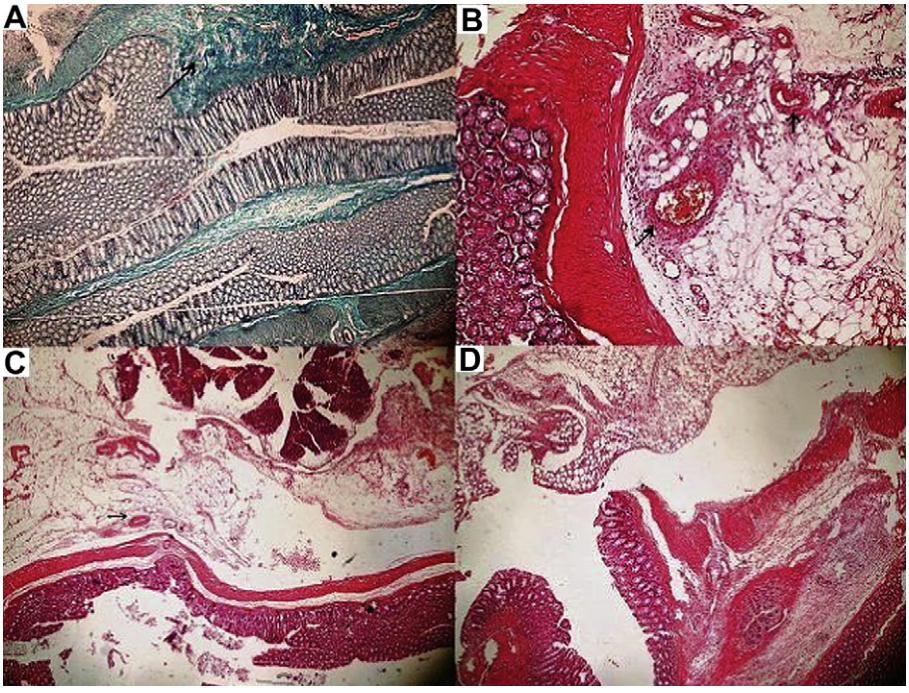

Fig. 3. A: Dense collagen deposition (black arrow) is shown in the anastomotic area. The blue area is connective tissue (Masson Trichrome × 40) (Group 4). B- Many newly-formed vessels are seen (HE × 100) (Group 3). **C-** Slight inflammation and angiogenesis are seen (HEx40) (Group 1). **d-** Dense inflammation and angiogenesis can be seen (HEx40) (Group 5).

involved in the regulation of angiogenesis during wound healing.[5,6] Blood vessels respond to higher VEGF levels with vasodilation and increased permeability and neovascularisation begins to take place in its presence.[4] In order to determine the efficiency of growth factor delivery, we studied VEGF and FGF tissue levels in all groups. We found significantly higher VEGF levels in groups 3 and 5 than in the control group. FGF level was increased in groups 3–5, which was unexpected in the case of group 3 (VEGF plasmid). A potential explanation of this result may be that FGF stimulates endothelial cells, which then upregulate VEGF.[10]

When comparing the histological parameters, necrosis, angiogenesis and fibroblastic activity were similar to the previously mentioned results. This study showed that delivery of growth factors increased collagen production and angiogenesis and decreased necrosis in groups 3–5. The necrosis score was highest (2.8) in the control group (group 1), whereas the lowest score was found in group 5 (1.2). This experimental group was associated with newly-formed capillary networks. In addition, when comparing rates of angiogenesis, rates were higher in groups 3–5, where these increases were statistically significant. Neovascularisation is a crucial step in the wound healing process.[23,24] Angiogenesis is the growth of new vessels to meet the demand for O_2, nutrition and inflammatory cell for wound healing.[25] Several angiogenic activators, including members of the VEGF and FGF gene families and various inhibitors of angiogenesis, have been described. The balance between angiogenic activators and inhibitors results in limited new blood vessel growth in the majority of tissues.[26] Animal studies have shown that the administration of VEGF restores impaired angiogenesis found in diabetic ischemic limbs.[10] There are currently only a handful of gene therapy clinical trials investigating the application of growth factors to chronic wounds. Margolis[27] and colleagues have shown that delivery of a recombinant adenovirus expressing PDGF increased the healing of diabetic foot ulcers and chronic venous ulcers. Both recombinant and homologous/autologous growth factors could be used clinically, where autologous growth factors are harvested from the patients' own platelets. This approach allows for treatment with patient-specific factors in a physiological concentration ratio.[3]

Our results demonstrate a strong correlation between histological findings and bursting pressure. In addition, there is an important link between collagen deposition and hydroxyproline level. We found higher hydroxyproline levels and collagen deposition in experimental groups 4 and 5. In group 3, although the hydroxyproline level was higher than in the control group, collagen deposition was not found to be significantly higher. In conclusion, this is the first study that used growth-factor-mediated gene therapy for the healing of ischemic colonic anastomosis. This therapy augmented the levels of hydroxyproline and bursting pressure. Moreover, this method significantly accelerated many of the histological parameters of ischemic colonic anastomosis. Combinations of growth factors may have a synergistic effect on ischemic wound healing processes. Growth-factor-mediated naked DNA therapy can be effectively used in increasing ischemic wound healing anastomosis.

Conflicts of interest

No.

Sources of funding

No.

Ethical approval

Yes, Istanbul Faculty of Medicine Department of Experimental, 042008, 2008. The study protocol was approved by the Institutional Animal Care and Use Committee at Istanbul University. 27.08.2009, No:96/107.

Author contribution

GA, AP, MA: the conception and design of the study; **SA, OK, DU:** acquisition of data, or analysis and interpretation of data; **Kemik O, AC, NA, AK, GK:** drafting the article or revising it critically for important intellectual content; **SK, OK:** final approval of the version to be submitted.

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