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The effects of Pycnogenol® on colon anastomotic healing in rats given preoperative irradiation☆

K. Cumhur Değer [a,*], Ahmet Şeker [a], İlter Özer [a], E. Birol Bostancı [a], Tahsin Dalgıç [a], Müge Akmansu [b], Özgür Ekinci [c], Uğur Erçin [d], Ayşe Bilgihan [d], Musa Akoğlu [a]

[a] Department of Gastroenterological Surgery, Turkiye Yuksek Ihtisas Teaching and Research Hospital, Ankara, Turkey
[b] Department of Radiation Oncology, Gazi University Medical Faculty Hospital, Ankara, Turkey
[c] Department of Pathology, Gazi University Medical Faculty Hospital, Ankara, Turkey
[d] Department of Biochemistry, Gazi University Medical Faculty Hospital, Ankara, Turkey

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ABSTRACT

Pycnogenol® has excellent radical scavenging properties and enhances the production of antioxidative enzymes which contributes to the anti-inflammatory effect of the extract. Irradiation delivered to the abdominal region, typically results in severe damage to the intestinal mucosa. The effects of ionizing radiation are mediated by the formation of free radicals through radiolysis. Irradiation has local effects on tissues. These local effects of irradiation on the bowel are believed to involve a two-stage process which includes both short and long term components. In our study we aimed to investigate the short term effects of Pycnogenol® on the healing of colon anastomoses in irradiated bowel. Sixty male Wistar-Albino rats were used in this study. There were three groups: Group I, control group ($n = 20$); group II which received preoperative irradiation ($n = 20$); group III which received per oral Pycnogenol® before irradiation ($n = 20$). Only segmeter colonic resection and anastomosis was performed to the control group (Group I). The other groups (Group II, III) underwent surgery on the 5th day after pelvic irradiation. On postoperative days 3 and 7, half of the rats in each group were sacrificed and then relaparotomy was performed. There was no statistical difference between groups with respect to biochemical parameters. Bursting pressure was significantly higher in the Control and Group III compared with the Group II. In conclusion, the present study showed that preoperative irradiation effect negatively on colonic anastomoses in rats by means of mechanical parameters and administration of Pycnogenol® preoperatively ameliorates this unfavorable effect.

1. Introduction

Pycnogenol® is a standardized extract from French maritime pine bark (*Pinus pinaster*) containing a mixture of procyanidins. Major constituents of Pycnogenol® are polyphenols, specifically monomeric and oligomeric units of catechin, epicatechin and taxifolin. It is well documented that polyphenols comprise a wide area of natural substances of plant origin and almost all of them exhibit a marked antioxidant activity.[1,2] The maritime pine bark extract Pycnogenol® has excellent radical scavenging properties and enhances the production of antioxidative enzymes which contributes to the anti-inflammatory effect of the extract.[3,4] Pycnogenol®, containing procyanidins and other phenolic constituents, recycles oxidized ascorbate. Pycnogenol® constituents have been shown to display high affinity to collagen and elastin and inhibit their enzymatic hydrolysis by matrix metalloproteinases. The ability of Pycnogenol® to reduce oxidized ascorbate will probably extend the activity of the vitamin in the wound vicinity, supporting collagen formation. The pronounced binding of Pycnogenol® constituents to collagen (and elastin) and their significant inhibitory effect on matrix metalloproteinases (MMPs) are likely to represent a key function in wound healing.[5]

Anastomotic leakage following colorectal resection and primary anastomosis is a major clinical problem. Numerous risk factors have been implicated as predisposing for anastomotic leaks. Schrock

☆ This work designed at Turkiye Yuksek Ihtisas Teaching and Research Hospital, Ankara.

* Corresponding author.
E-mail addresses: cumhurdeger@hotmail.com (K.C. Değer), sekerahmet@hotmail.com (A. Şeker), ilterozer@yahoo.com (İ. Özer), ebb@yahoo.com (E.B. Bostancı), td@gmail.com (T. Dalgıç), akmansu@gmail.com (M. Akmansu), ozzyekinci@hotmail.com (Ö. Ekinci), ugurercin67@gmail.com (U. Erçin), ayseb@gazi.edu.tr (A. Bilgihan), makoglu@yahoo.com (M. Akoğlu).

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et al. performed a large retrospective analysis of factors relating to leakage of colonic anastomoses. Factors that were found to correlate with an increased leakage rate were older age, anemia, prior radiation therapy, intraperitoneal infection and anatomic level of anastomosis.[6] Irradiation delivered to the abdominal region, a common treatment for malignancies, typically results in severe damage to the intestinal mucosa, because of the high sensitivity of the gastrointestinal tract to ionizing radiation.[7] The effects of ionizing radiation are mediated by the formation of free radicals through radiolysis, which are highly reactive, removing hydrogen atoms from fatty acids, causing lipid peroxidation and consequently cell death. A number of antioxidant agents, the radioprotective drugs, have been used in attempting to minimize the side effects caused by radiotherapy in the abdominal region or in nuclear accidents affecting the whole body. In a study on rats, preoperative vitamin A supplementation protected against impaired colonic healing caused by preoperative radiation therapy.[8] In another study Flávia et al. demonstrated that French maritime pine bark extract Pycnogenol® could significantly lower tissue damage resulting from x-irradiation.[9]

The aim of this study was to investigate the early effects of Pycnogenol® on the healing of colon anastomoses in irradiated bowel.

2. Materials and methods

The study was approved by the Ethics Committee on Animal Experimentation of the Faculty of Medicine of Ankara University (protocol no. 2011-105-386). Sixty male Wistar-Albino rats weighing between 180 and 220 g were used in this study. The animals were housed in stainless steel cages under controlled temperature (23 °C) and humidity conditions, and 12 h of dark/light cycles. All rats were clinically healthy and were fed with standard laboratory food and water. There were three groups: Group I, control group ($n = 20$); group II which received preoperative irradiation ($n = 20$); group III which received per oral Pycnogenol® before irradiation ($n = 20$).

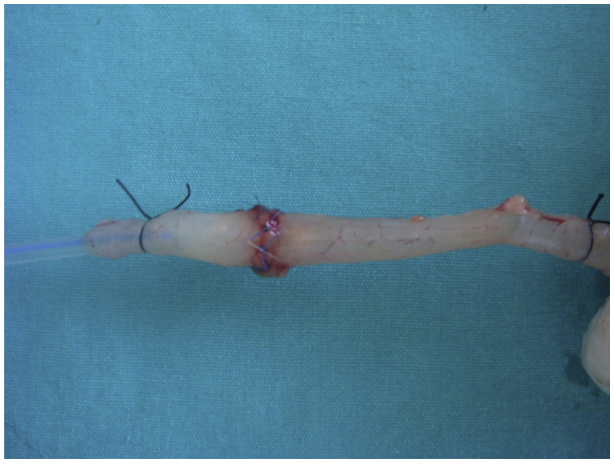

Fig. 2. Assessment of anastomotic colonic segment bursting pressure.

Only segmeter colonic resection and anastomosis was performed to the control group (Group I). The other groups (Group II, III) underwent surgery on the 5th day after a single dosage of pelvic irradiation. On postoperative days 3 and 7, half of the rats in each group were anesthetized with ketamine hydrochloride (75–100 mg/kg) and xylazine (10 mg/kg) intramuscularly and sacrificed by intracardiac puncture and then relaparotomy was performed for in vitro analytic procedures (Fig. 1). 3 cm of colonic segment, including the anastomotic area was resected and bursting pressure

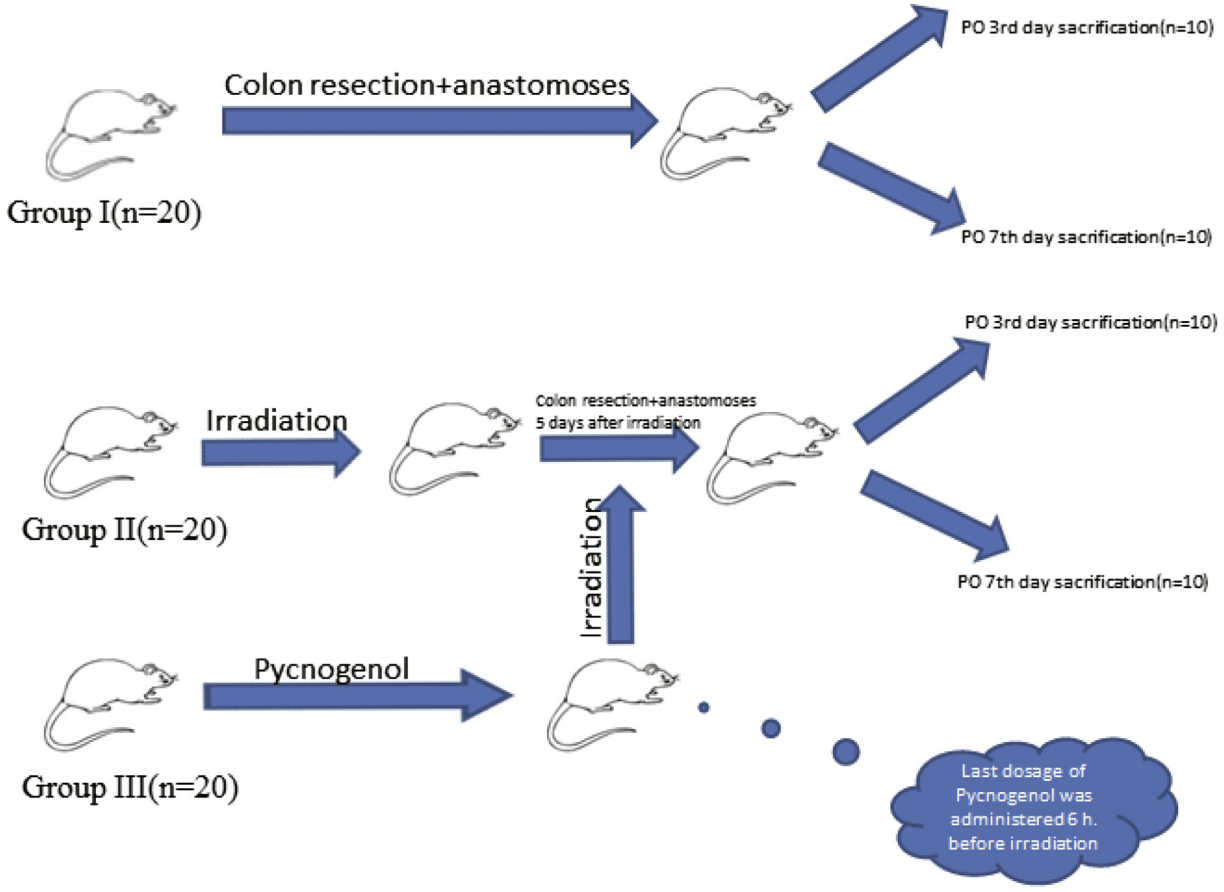

Fig. 1. Schematic diagram of the study design.

was measured in vitro for every anastomotic segment. For this purpose, the segments were infused 2 mL/min with 0, 9% NaCl (Fig. 2). The maximum pressure recorded immediately before sudden loss of pressure was taken as the bursting pressure. The site of the rupture (within or outside the anastomosis) was noted.

After recording the bursting pressure, the anastomosis was opened longitudinally and divided into three equal longitudinal segments. One segment was placed in 10% formalin for histological assessment. The second segment of the anastomosis site was frozen in liquid nitrogen and stored at −80 °C until measurement of anastomotic tissue malondialdehyde (MDA) and advanced oxidation protein products (AOPP) levels were measured, the third segment of the anastomosis was prepared as the same for measurement of anastomotic tissue hydroxyproline (HP) contents.

2.1. Drug administration

When related literature was taken into account, 200 mg/kg Pycnogenol® was administered three times, starting 3 days prior to x-irradiation. The final administration was carried out 6 h before irradiation to maintain a sufficient plasma concentration. It was demonstrated in a study that applied dosages of Pycnogenol® as described above provide significant protection against deleterious effects from whole body ionizing radiation on the intestinal mucosa.[9] Pycnogenol® was administered orally to mice in a dosage of 150 and 200 mg/kg body weight, which is in the same range as in our experiments.

2.2. Irradiation technique

Before intramuscular administration of ketamine HCl (10 mg/kg) (Ketalar) was provided for anesthetization, rats were grouped into subgroups that each contained 5 rats. Each group underwent half body radiation according to the source axial distance (SAD) technique under the simulator (Verasim II, GE Medical Systems, Waukesha, Wis). All groups were exposed to a single dosage of 485 cGy g-ray (Cobalt 60 Teletherapy Theratron 780 C, Atomic Energy of Canada Ltd., Kanata, Ontario, Canada) in the same position, with openings above and below opened parts (parallel). Rats were put into cages and started to feed after 4 h.

2.3. Operative procedure

The same operative procedure was performed in all groups by the same surgeon. All surgical procedures were performed under sterile conditions; after shaving the frontal abdominal wall, 2.5% povidone-iodine was used for skin disinfection and rats were anesthetized with ketamine hydrochloride (75–100 mg/kg) and xylazine (10 mg/kg) intramuscularly. The abdomen was entered through a 3 cm mid-line incision, 0.5 cm of colon 3 cm proximal of the peritoneal reflection was resected, and an end-to-end anastomosis was made using eight inverting interrupted sutures with 5/0 polypropylene (Ethicon). Muscles of the front abdominal wall and skin were closed by continuous suture with 3/0 silk (Ethicon) and rats were kept in their cages.

2.4. Determination of tissue HP, MDA and AOPP levels

Anastomotic HP content was measured according to the method of Jamall et al.[10] Briefly, 25 μg of homogenate taken from hydrolyzation was lyophilisated and dissolved in the 1 mL 50% (vol/vol) isopropyl alcohol. Ten minutes later, chloramine T was added to these samples. Next, the samples were incubated for 90 min at 50 °C after adding 1 mL Erlich's reagent. Absorbance was measured at 560 nm, and the amount of HP was calculated from a standard curve constructed using HP at concentrations 0.25–40 μg/mL. Results were expressed as μg/mg tissue.

Spectrophotometric determination of AOPP levels was performed by modification of Witko's method.[11] Samples were prepared in the following way: Two hundred microliters of supernatant were diluted 1:3 in PBS, 100 mL of 1.16 mol/L potassium iodide (KI, Sigma) were then added to each tube, 2 min later followed by 200 mL of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 1200 mL of PBS, 100 mL of KI, and 200 mL of acetic acid. Concentrations of AOPP were calculated by using the extinction coefficient of 26 mM21 3 cm21.

The level of MDA in the homogenate from each group was measured using the method of Uchiyama et al.[12] Determination of malondialdehyde precursor in tissues by thiobarbituric acid test was as follows. In brief, 250 μl of 10% homogenate of the tissue sample was added to 1.5 ml of 1% H_3PO_4 and 0.5 ml of 0.6% tert-butyl alcohol (aqueous solution), and then the mixture was stirred and heated on a boiling water bath for 45 min. After cooling, we added 2 ml of n-butanol, and the mixture was shaken and the butanol layer was separated by centrifugation. Optical density of the butanol layer was determined to be 535 and 520 nm, and the optical density difference between the two determinations was calculated (as the tert-butyl alcohol value). MDA concentrations were expressed as nanomoles per gram of tissue.

2.5. Histology

Perianastomotic colonic segments were sampled for examination by an expert pathologist blinded to experimental groups. A segment of each anastomotic ring was removed for histological examination and fixed in 10% formaldehyde. The samples for histology were dehydrated and embedded in paraffin. The paraffin blocks were cut at 4 μm thickness and serial sections were prepared for staining with hematoxylin–eosin (H&E). Histological changes of anastomotic wound healing, granulation tissue development, local inflammatory response, and neovascularization were determined according to Houdart et al. and Hutschenreiter et al. parameters as modified by Garcia et al.[13–15] (Table 3).

2.6. Statistical analysis

Data analysis was performed by using SPSS for Windows, ver. 11.5. Shapiro Wilk test was used to determine whether the distributions of continuous variables were normal or not, the homogeneity of variables was determined by Levene test. Data were shown as mean ± standard deviation or median (minimum–maximum), where applicable. Bonferroni adjusted Kruskal Wallis test was applied for determining the differences among more than two dependent groups. When the *P* value from the one-way ANOVA or Kruskal Wallis test was statistically significant to determine which groups differ from others by using *post hoc* Tukey multi comparison test was used. A P value < 0.025 was considered statistically significant. However, all possible multiple comparisons, the Bonferroni adjustment was applied for controlling Type I error.

3. Results

During the experimental procedure, one animal died on 2nd postoperative day in the control group. The cause of death was from the aggressive behavior of the animal. Post mortal exploration revealed no anastomotic leakage from the anastomotic site. No mortality was observed in the other two groups. While dissecting perianastomotic region there was much more omental tissues surrounding colon but however it was easy to dissect away those structures in Group II. There was no macroscopic anastomotic leakage in all groups.

3.1. Anastomotic strength

The strength of the anastomosis was determined by measuring colonic bursting pressure. All ruptures occurred at the anastomotic site on postoperative 3rd (D 3) anD 7th (D 7) day in Group II. However, on postoperative D 7, ruptures occurred away from the anastomosis in three of rats in the control group and in four of rats in the Group III. Bursting pressure was significantly higher in the Control and Group III compared with the Group II ($p < 0.001$) (Table 1).

3.2. Assessment of HP, MDA, AOPP levels in the Control Group, Group II and Group III on postoperative D 3

There was no significant difference between the Control Group, Group II and Group III in terms of HP ($p = 0.068$) and AOPP ($p = 0.827$) levels. MDA levels were higher in the Group II (216.6 ± 87.7) than the Control group (121.2 ± 48.2) and Group III (162.5 ± 108.4). However, the difference between groups was not statistically significantly ($p = 0.034$).

Table 1
Bursting pressure (mmHg) on postoperative D 3 and D7.

Days	Control Group	Group II	Group III	*p* value[a]
D 3	70.1 ± 10.0^{b}	$55.9 \pm 6.2^{b,c}$	66.8 ± 9.8^{c}	0.007
D 7	185.4 ± 23.6^{b}	$124.9 \pm 20.2^{b,c}$	171.9 ± 31.8^{c}	<0.001

[a] The comparisons within days, Kruskal Wallis test, according to the Bonferroni adjustment $P < 0.025$ was considered statistically significant.
[b] The difference between Control and Group II was found statistically significant ($p < 0.001$).
[c] . The difference between Group II and III was found statistically significant ($p < 0.001$).

Table 2
Biochemical parameters of each group on postoperative D 3 and D 7.

	Control Group	Group II	Group III	*p* value[a]
MDA				
D 3	121.2 ± 48.2	216.6 ± 87.7	162.5 ± 108.4	0.034
D 7	267.4 ± 151.7	184.3 ± 112.5	271.4 ± 143.4	0.381
AOPP				
D 3	6.1 ± 2.0	6.0 ± 3.0	7.6 ± 5.4	0.827
D 7	9.5 ± 6.7	4.1 ± 2.1	5.8 ± 3.6	0.133
HP				
D 3	0.34 ± 0.06	0.45 ± 0.26	0.27 ± 0.16	0.068
D 7	0.37 ± 0.12	0.50 ± 0.21	0.36 ± 0.17	0.186

[a] One-way ANOVA, according to the Bonferroni adjustment $P < 0.025$ was considered statistically significant.

3.3. Assessment of HP, MDA, AOPP levels in the Control Group, Group II and Group III on postoperative D 7

There was no significant difference between the Control Group, Group II and Group III in terms of HP ($p = 0.186$), MDA ($p = 0.381$) and AOPP ($p = 0.133$) levels (Table 2).

3.4. Histopathological evaluation of anastomotic layer on postoperative D 3 and D 7

In terms of mucosal anastomotic reepithelialization and muscle layer destruction there were no statistical significant difference between groups on postoperative D 3 and D 7 ($p > 0.017$). On postoperative D 3 the difference between each groups was not significant in terms of granulation tissue formation but on D 7 it was significantly higher in the Group II and Group III when compared to the Control Group ($p < 0.001$). Anastomotic wound inflammatory infiltration was similar on postoperative D 3, it was significantly higher on postoperative D 7 in Group III when compared to Control Group and Group II($p < 0.001$) (Table 4). H&E-stained sections of colonic anastomoses are shown in Fig. 3.

Table 3
Parameters of histologic changes of anastomotic wound healing, granulation tissue development, and local inflammatory response.[13–15]

1. Mucosal anastomotic reepithelialization	
Grade 0	Absence of epithelialization on the anastomotic line
Grade 1	Incomplete coating of the anastomotic wound with a single layer of cells
Grade 2	Complete coating of the anastomotic wound with a single layer of cells
Grade 3	Complete reepithelialization with glandular epithelium

2. Inflammatory granuloma and granulation tissue formation				
	Inflammatory cell presence	Neovascularization	Fibroblasts	Fibrosis formation
Grade 1	Absence	Absence	Absence	Absence
Grade 2	Slight	Slight	Slight	Slight
Grade 3	Mild	Mild	Mild	Mild
Grade 4	Intense	Intense	Intense	Intense

3. Muscle layer destruction			
	Ischemic necrosis	Muscle layer continuity	Inflammatory infiltration
Grade 1	Absence	Complete interruption	Absence
Grade 2	Slight	Muscle synechia	Slight
Grade 3	Mild	Complete restitution	Mild
Grade 4	–	–	Intense

4. Anastomotic wound inflammatory infiltration				
	Neutrophils	Lymphocytes	Histiocytes	Giant cells
Grade 1	Absence	Absence	Absence	Absence
Grade 2	Slight	Slight	Slight	Slight
Grade 3	Mild	Mild	Mild	Mild
Grade 4	Intense	Intense	Intense	Intense

Table 4
The results of histopathological grading.

Variables	Control Group	Group II	Group III	*P* value[a]
Mucosal anastomotic reepithelialization				
Day 3	2 (1–3)	2.5 (1–3)	1 (1–3)	0.439
Day 7	1 (1–3)	1 (0–3)	1.5 (0–3)	0.807
Inflammatory granuloma and granulation tissue formation				
Day 3	2 (2–3)	3 (2–4)	3 (2–3)	0.672
Day 7	2.5 (2–3)[b,c]	3 (2–4)[b]	4 (2–4)[c]	0.004
Muscle layer destruction				
Day 3	3 (2–3)	3 (2–3)	3 (2–3)	0.806
Day 7	2 (1–3)	2 (1–3)	2 (1–4)	0.805
Anastomotic wound inflammatory infiltration				
Day 3	2 (1–3)	3 (2–4)	3 (2–3)	0.204
Day 7	3 (2–4)[c]	3 (2–4)[d]	4 (3–4)[c,d]	0.018

[a] P value < 0.025 was considered statistically significant according to the Bonferroni adjusted Kruskal Wallis test.
[b] the difference between the Control Group and Group II was statistically significant ($p < 0.001$).
[c] the difference between the Control Group and Group III was statistically significant ($p < 0.001$).
[d] the difference between Group II and Group III was statistically significant ($p < 0.001$).

4. Discussion

X-irradiation is commonly referred to as ionizing radiation, involving the generation of free radicals from radiolysis, such as hydroxyl radicals, which are largely responsible for the significant tissue damage. Acute high doses are known to particularly burden tissues with rapid cell renewal, causing bone marrow aplasia and structural alteration and dysfunction of the mucosa.[7] Doses of RT were attempted with the goal of determining how much is needed to achieve mucosal damage but not mortality in rats. Toward this end, it can be seen in the literature that local (abdominal) single-dose RT of 1000–1100 cGy has been used for intestinal damage in experimental rat models.[16–18] A single dose (485 cGy) less than 675 cGy, which is near the 50% lethal dose for gastrointestinal damage in humans, was preferred.[19] Irradiation has local effects on tissues. These local effects of irradiation on the bowel are believed to involve a two-stage process which includes both short- and long-term components.[20] Recovery from the short-term effects of irradiation can occur between 7 days and 1 month after treatment, depending on the dose, and long-term complications may develop anywhere from 1 month to 20 or more years after treatment. The short-term changes are manifested histologically by loss of the intestinal epithelial cell lining, disappearance of mitotic figures, and edema of the submucosa and muscularis. Usually, there is marked improvement 7–9 days after treatment, with the bowel appearing histologically near-normal by 22 days after treatment.[21] Long-term changes cause fibrosis, stricture, and ischemia secondary to hyalinization of blood vessels, which often become manifest when resting cells (capillary endothelial cells or fibroblasts) are stimulated to divide during normal wound repair.[22,23]

Leakage of anastomosis after colorectal resection is a serious problem in gastrointestinal surgery. After the introduction of preoperative irradiation, conflicting results have been reported on the incidence of anastomotic leakage.[24–26] In several animal[8,27,28] and clinical[29,30] studies, decreased anastomotic strength or higher incidence of anastomotic leakage after irradiation was demonstrated. Anastomotic bursting pressure is a reliable and objective parameter for colonic tissue healing.[31–33]

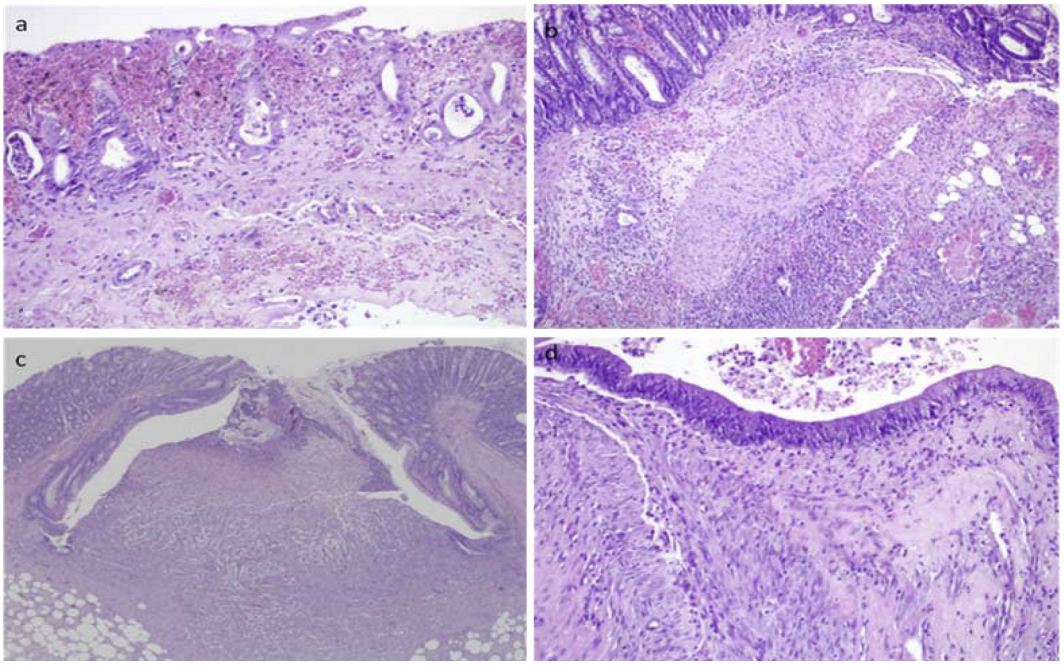

Fig. 3. Anastomotic histology. H&E-stained sections of colonic anastomoses collected on day 3 (a) Group II; (b) Group III and day 3; (c) Group II and day 7; (d) Group III and day 7.
a) Incomplete epithelization on the surface, areas characterized with new crypt formation and marked ischemic changes in the lamina propria. (H&E, ×200).
b) Partial muscle layer discontinuity and inflammation by mild neutrophil infiltration with mostly regenerated mucosa beneath. (H&E, ×100).
c) Deep ulceration with complete interruption of the muscle layer, intense inflammation and active granulation tissue. (H&E, ×40)
d) Anastomotic mucosal layer demonstrating intensive granulation tissue with seldom crypts. (H&E, ×200).

In this study we evaluated the short-term effects of irradiation on colonic anastomoses in rats that Pycnogenol® was administered preoperatively.

We found out that the estimated bursting pressures were lower in group II. So according to our study, we can say that irradiation delivered to the abdominal region decreases the bursting pressure, however this decrease did not result with any anastomotic failure. Also preoperative administration of Pycnogenol® had improved this negative effect. It is well documented that Pycnogenol® has excellent radical scavenging properties and exhibit a marked antioxidant activity.[1–4] However, in our study this property of the extract did not show any difference in terms of biochmemical parameters among groups, although MDA levels were lover in group III compared to Group II on postoperative 3rd day, but this result did not reach statistical difference. So we can't explain the ameloriative effect of Pycnogenol® by this well-known antioxidant effect and radical scavenging properties. As mentioned above, in an experminatal study, it was shown that Pycnogenol® constituents to collagen (and elastin) and their significant inhibitory effect on matrix metalloproteinases (MMPs) were likely to represent a key function in wound healing.[5] As a limitation of our study MMPs levels could be assesed to extrapolate the possible underlying mechanism of this ameliorative effect of the extract.

During the first 3–5 days after anastomosis, collagenolysis exceeds collagen synthesis and the balance shifts, favoring the former, resulting in loss of anastomotic strength with minimal values after approximately 3 days.[34,35] During this early phase of the healing process, known as inflammatory stage, anastomotic strength and integrity depend on the suture-holding capacity of the submucosa and anastomosis is theoretically under the greatest risk for leakage. Thereafter, wound strength increases rapidly, between the fifth and seventh days after surgery, collagen synthesis peaks during proliferative phase, and the healing process is dominated by the formation of a new matrix.[36] Therefore, the 3rd and the 7th postoperative days were used to evaluate the early and late anastomotic wound-healing process in our study.

To measure the HP level is an indirect way to evaluate the collagen content and is a useful method for biochemical tissue healing process. In the present study we didn't show any statistical difference between each group in the postoperative 3rd anD 7th days. There are several results have been reported similar and different from this study. Biert et al. like our study showed no statistical difference in the HP levels.[37] Kuzu et al. reported that the MPO, HP and bursting pressure levels were lower in the postoperative 3rd and 7th day in the irradiated rats' colonic anastomoses.[38]

Wound healing involves the coordinated action of several cell types and biochemical events. Anastomotic healing begins with a strong inflammatory reaction. The first cells to act are granulocytes, which appear within 3 h after the wound is created. Next, monocytes, macrophages, and lymphocytes appear in the wound. These cells reflect the immune reaction to tissue injury. Granulocytes and lymphocytes almost disappear in the later stages of wound healing. The local vascular response to trauma results in edema and necrosis in the anastomotic area during the first postoperative week.[39–41]

In our study mucosal anastomotic reepithelialization score was higher in Group III compared to the Control Group and Group II although this finding did not reach statistical difference. Anastomotic wound inflammatory infiltration was significantly higher on postoperative D 7 in Group III. This finding suggests that Pycnogenol® will probably also assist the first, i.e. the inflammatory, stage of the wound-healing process. During this phase, neutrophils and monocytes at the wound site carry out various tasks, such as the removal of bacteria to prevent the wound from becoming infected. Pycnogenol® was recently discovered to have bacteriostatic activity against a broad range of Grampositive and negative bacteria, as well as Candida albicans, with minimum inhibitory concentrations of 20–100 μg/mL[42]

In conclusion, the present study showed that preoperative irradiation effect negatively on colonic anastomoses in rats by means of mechanical parameters and administration of Pycnogenol® preoperatively ameliorates this unfavorable effect. Accoring to our study we can't explain the ameliorative effect of Pycnogenol® in terms of bursting pressure with well-known antioxidant feature. There may be some other mechanisms to explain this effect of the extract. Further studies are needed to reach a definite conclusion.

Ethical approval

The study was approved by the Ethics Committee on Animal Experimentation of the Faculty of Medicine of Ankara University (protocol no. 2011-105-386).

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Author contribution

K. Cumhur Değer, MD study design.
Ahmet Şeker, MD study design.
İlter Özer, MD writing.
E. Birol Bostancı, MD data analysis.
Tahsin Dalgıç, MD data collection.
Müge Akmansu, MD irradiation study.
Özgür Ekinci, MD pathological study.
Uğur Erçin, MD biochemical study.
Ayşe Bilgihan, MD biochemical study.
Musa Akoğlu, MD data analysis.

Conflicts of interest

There are no conflicts of interest.

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