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Original Article

Effects of ghrelin on colonic anastomosis following radiotherapy

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Abstract: Background: Ghrelin has been reported to have properties such as inducing proliferation, migration and angiogenesis of vascular endothelial cells and has anti-inflammatory effects which may help wound healing in anastomosis following neoadjuvant radiotherapy. Materials and methods: Rats were randomized into 4 groups: control group (1) underwent colonic anastomosis without treatment; ghrelin group (2) which received a single dose of ghrelin following colonic anastomosis; radiation group (3) which recieved a single dose of radiation 5 days before anastomosis; radiation + ghrelin group (4) which received a single dose of radiation 5 days before anastomosis and ghrelin following anastomosis. Anastomotic bursting pressures of the rats which were sacrificed 7 days after surgery were measured. The anastomotic segment was extracted for tissue hydroxyproline, luminol and lucigenin measurements, and histopathological examination as well. Blood samples were obtained for measurement of TNF-α and IL-1ß plasma levels. Results: Bursting pressures of anastomosis and hydroxyproline levels were significantly higher in control and radiation + ghrelin groups than in radiation group. Tissue luminol and lucigenin levels as well as plasma TNF-α and IL-1ß levels were significantly lower in control and radiation + ghrelin groups than in radiation group. Histopathological assessment revealed that administration of ghrelin resulted in regression of inflammation which was severe in radiation group. Conclusion: The adverse effects of radiotherapy on mechanic and histological parameters of anastomosis healing were attenuated by ghrelin therapy by reducing plasma proinflammatory cytokines and tissue reactive oxygen radicals.

Keywords: Ghrelin, radiotherapy, anastomosis, oxidative stress, healing

Introductıon

Postoperative local recurrences after surgery are still common despite the introduction of total mesorectal excision for the treatment of rectal cancer [1]. Neoadjuvant chemoradiotherapy or radiotherapy alone has become a standard treatment of locally advanced rectal tumors to avoid recurrences and also enables the preservation of the anal sphincters in cases of lower rectal tumors [2-4]. Anastomosis leakage is an important cause of morbidity and mortality after colorectal surgery. The risk of colorectal anastomotic dehiscence is greater as compared to other segments of the gastrointestinal tract because of its higher bacterial content and scant blood supply especially in elder patients with poor physical condition [5]. Preoperative radiotherapy combined with TME may disrupt the healing of colorectal anastomosis even more. Studies have shown that radiotherapy may delay anastomotic healing [6, 7] and actually may increase the anastomotic leakage rate compared to the control groups [8, 9]. Experimental studies have shown that preoperative radiotherapy may decrease the anastomotic bursting pressure and the level of hydroxyproline in the anastomosis [10].

Healing of the colorectal anastomosis involves a complex interaction of peptide growth factors and collagen turnover [5]. Ghrelin is a peptide,

predominantly produced by the stomach, which is an endogenous ligand of growth hormone stimulating receptor and has influence on appetite and sleep, cardiovascular, gastrointestinal and endocrine systems. It has also been demonstrated in animal models of inflammatory bowel diseases that ghrelin potentiates proliferation, migration and angiogenesis of vascular endothelial cells and has anti-inflammatory properties [11]. Ceran *et al.* hypothesized that ghrelin may have positive effects on colonic anastomosis, partly related to increased growth hormone secretion which may promote angiogenesis and consistent with this hypothesis and they found that bursting pressures were effectively increased following ghrelin administration [12]. Yu *et al.* have reported similar benefits of ghrelin in rats which underwent subtotal gastrectomy [13]. The aim of the present study was to investigate the effects of ghrelin on the healing of anastomosis in a rat model of post radiation therapy colonic anastomosis.

Materials and methods

This study was approved by Local Ethical Committee of Animal Experiments of Marmara University (protocol no: 73.2012.mar). Thirty-two adult male Wistar rats weighing 200-250 g were used in the present study. All animals were kept in a temperature-controlled room (22 ± 2°C) with a 12-hour light-dark cycle with free access to water and standard laboratory chow.

A single dose of ghrelin (Sigma-Aldrich Corp, St. Louis, MO, USA) was administered subcutaneously at a dose of 10 ng/kg 30 min after the surgery. Rats were exposed to a single dose of (800 rad) radiation at a source-axis distance of 100 cm, 5 days before the surgery.

Rats were randomized into 4 groups. Group 1: Control group; Colonic anastomosis without administration of ghrelin and radiation; Group 2: Ghrelin group; Ghrelin + colonic anastomosis; Group 3: Radiation group; Radiation + colonic anastomosis; Group 4: Radiation + Ghrelin group; Radiation + Ghrelin + Colonic anastomosis.

Surgical procedure

All rats were fasted for 12 hours prior to the surgery. Anesthesia was induced by intramuscular administration of 50 mg/kg ketamine (Ketalar, Pfizer, Turkey) and 10 mg/kg xylazine HCl (Rompun, Bayer, Turkey). Abdominal hair was shaved, skin was sterilized with 10% povidone iodine, and laparotomy by a 3-cm midline incision was performed under general anesthesia. Sigmoid colon was transected 3 cm above the peritoneal reflection without disrupting perfusion of the mesocolon. Afterwards, anastomosis was performed using interrupted 6/0 polypropylene (Prolene, Ethicon) sutures in a single layer, end to end manner. 8 sutures were used for each anastomosis. All procedures were performed by a single surgeon. Fascia and skin were closed using continuous 3/0 polypropylene (Prolene, Ethicon). Standard laboratory chow and water was given 12 hours after the operation. Rats were sacrificed on postoperative day 7 and peritoneal cavity was exposed with a midline incision.

Irradiation technique

Before irradiation, rats were anesthetized with a single dose of 10 mg/kg ketamine (Ketalar, Pfizer, Turkey) and 1 mg/kg xylazine (Rompun, Bayer, Turkey), administered intramuscularly. Anesthetized rats in groups 3 and 4 were placed on a styrofoam block. Target fields of 5×5 cm in the pelvic regions with 2 cm in depth were irradiated with a single dose of 800 rad and a source axis distance of 100 cm using Siemens ONCOR Impression Plus (Siemens Medical Systems, Concord, CA) as described previously.

The anastomotic colonic parts were resected en bloc with 4 cm of bowel on each side. To measure bursting pressure, an 18 gauge intraluminal catheter was inserted and the segments were infused (6 ml/min) with saline containing methylene blue via infusion pump. The bursting pressure was identified when sudden loss of pressure and leakage was observed.

Measurement of hydroxyproline level

After measuring bursting pressure, a 10 mm-wide ring of tissue, comprising the anastomosis, was removed. Half of this removed tissue was preserved at -20°C for later measurements of hydroxyproline, luminol and lucigenin levels and the other half was fixed in 10% formaldehyde for histopathological evaluation. The amount of hydroxyproline was determined as Jamall *et al*. described [14].

Table 1. Median levels of bursting pressures and tissue hydroxyproline, luminol and lusigenin and median levels of plasma TNF-α and IL-1β

	Group I	Group II	Group III	Group IV
Bursting pressure (mm/Hg)	135.4 ± 8.7**	165.0 ± 2.9***	104.4 ± 4.4	126.4 ± 4.3*
Hydroxyproline (mg/g protein)	0.38 ± 0.04*	0.65 ± 0.06***,++	0.13 ± 0.02	0.44 ± 0.08*
Luminol (rlu/mg)	117.1 ± 10.6***	98.5 ± 14.1***	219.3 ± 25.5	117.9 ± 16.6***
Lusigenin (rlu/mg)	127.2 ± 7.1***	103.5 ± 4.9***	277.5 ± 30.9	178.2 ± 15.1**
TNF-á (pg/ml)	47.2 ± 2.3**	43.8 ± 2.6**	64.2 ± 4.1	50.2 ± 2.6*
IL-1â (pg/ml)	342 ± 23**	305 ± 15**	445 ± 15	370 ± 13*

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus group III; ++$P < 0.01$ versus group I.

Chemiluminescence (CL) assay

Luminol and lucigenin chemiluminescences were measured as indicators of radical formation to assess the contribution of reactive oxygen species in irradiation induced colonic damage. Measurements were done by using Junior LB 9509 luminometer (EG&G Berthold, Germany). Specimens were put into vials containing PBS-HEPES buffer (0.5 M PBS containing 20 mM HEPES, pH 7.2). After the addition of enhancers, lucigenin or luminol, for a final concentration of 0.2 mM, reactive oxygen radicals were quantitated. Luminol detects a group of reactive species, such as, OH, H_2O_2, HOCl radicals, while lucigenin is selective for O^{-2} [15, 16]. Counts were obtained at 1 min intervals and the results were given as the area under curve for a counting period of 5 min. Counts were corrected for wet tissue weights and expressed as relative light units (rlu/mg tissue) [17].

Blood assays

Plasma levels of TNF-α and IL-1β were quantified according to the manufacturer's instruction using enzyme-linked immunosorbent assay (ELISA) kits (Biosource International, Nivelles, Belgium). The total antioxidant capacity in plasma was measured by using colorimetric test system (ImAnOx, cataloge no. KC5200, Immunodiagnostic AG, D-64625 Bensheim).

Histopathological assessment

For light microscopic analysis, the tissue samples were dehydrated in ascending alcohol series and embedded in paraffin wax following fixation in 10% buffered formalin for 48 hours. Sections with 5 μm of thickness were stained with hematoxylin-eosin (H&E) for general morphology. For electron microscopic investigation, the samples were fixed in 4% phosphate buffered gluteraldehyde (0.13 M and pH 7.4) for 4 hours and post-fixed with 1% OsO_4 for one hour, dehydrated in graded alcohol series, put into amyl acetate, dried with liquid CO_2 under pressure with critical point dryer (Bio-Rad E 3000) and covered with gold particles (Bio-Rad SC502). Sections were observed under a photomicroscope (Olympus BH 2, Tokyo, Japan) or a scanning electron microscope (SEM; Jeol 1200 JSM, Tokyo, Japan) by an experienced histologist, who was blinded to the experimental groups.

Statistical analysis

The results were analyzed by Kruskal-Wallis one way ANOVA with Tukey test using SPSS 18.0 for Windows (SPSS, Inc., Chicago, Illinois). $P < 0.05$ was considered statistically significant.

Results

Bursting pressures in control, ghrelin, radiation and radiation + ghrelin groups were 135.4 ± 8.7 mmHg, 165 ± 2.9 mmHg, 104.4 ± 4.4 mmHg and 126.4 ± 4.3 mmHg, respectively. Bursting pressure in radiotherapy group was significantly lower compared with control group and radiotherapy + ghrelin group ($P < 0.01$ and $P < 0.05$, respectively) (**Table 1**).

Hydroxyproline levels in ghrelin group were significantly higher than in control group ($P < 0.01$) whereas significantly lower in radiotherapy group than in control and radiotherapy + ghrelin groups ($P < 0.05$ for both) (**Table 1**).

Luminol levels in radiotherapy group were significantly higher than in control and radiotherapy + ghrelin groups ($P < 0.001$ for both). Similarly, lucigenin levels in radiotherapy group were significantly higher than in control and

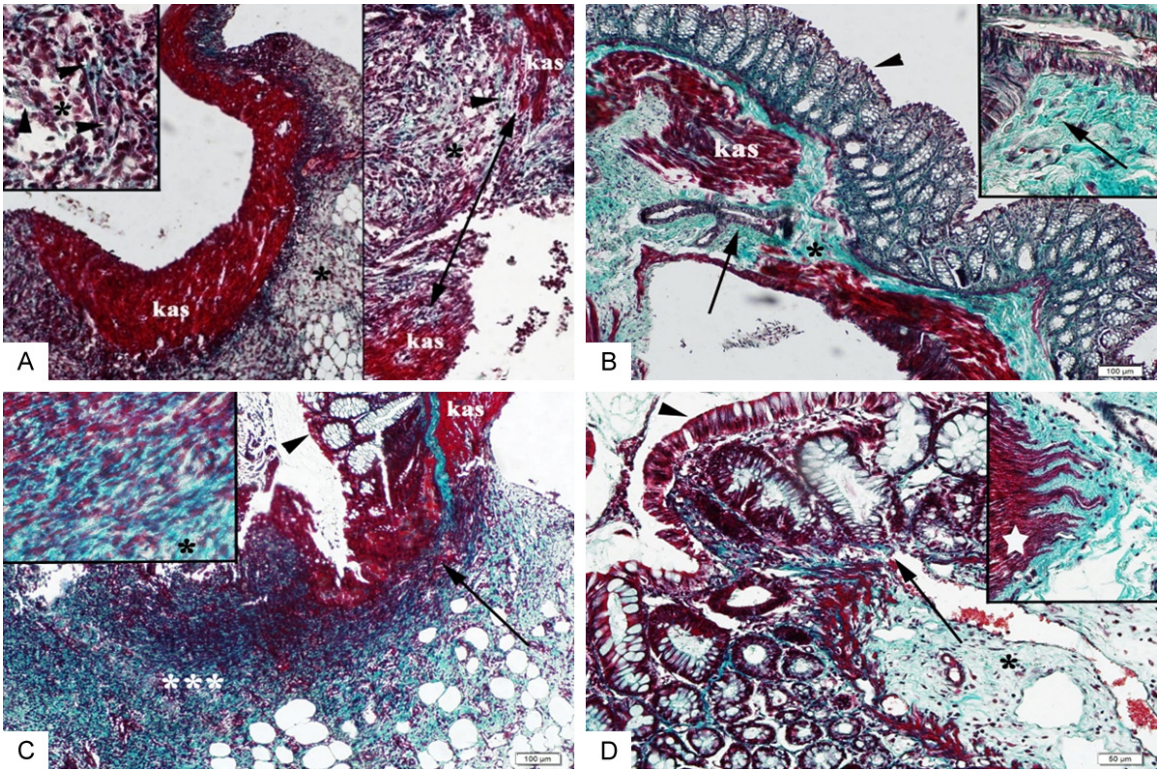

Figure 1. Anastomotic histology. H&E-stained sections of colonic anastomoses. A. Group I: Intense inflammation (*) and leukocyte recruitment (arrowheads) and reformation of muscle tissue along the anastomosis (double arrow). B. Group II: Reduced inflammation (arrows) and formation of collagen in lamina propria (*), regular epithelium (arrowhead). C. Group III: Intense leukocyte recruitment and inflammation (arrow), disrupted epithelium (arrowhead) and intense formation of collagen in lamina propria (white and black ***). D. Group IV: Anastomosis line (arrow) and regular muscle tissue (white star) and well-organized collagen tissue in lamina propria (*).

radiotherapy + ghrelin groups ($P < 0.001$ and $P < 0.01$, respectively) (**Table 1**).

Serum TNF-α levels in radiotherapy group were significantly higher than in control and radiotherapy + ghrelin groups ($P < 0.01$ and $P < 0.05$, respectively). Likewise, serum IL-1β levels in radiotherapy group were significantly higher than in control and radiotherapy + ghrelin groups ($P < 0.01$ and $P < 0.05$, respectively) (**Table 1**).

In histopathological assessment, inflammation along the incision line in control group (**Figure 1A**) was observed as decreased in ghrelin group (**Figure 1B**). In radiotherapy group, incision line was not distinct due to severe inflammation accompanied by necrosis (**Figure 1C**). Administration of ghrelin resulted in regression of inflammation and morphologically distinct incision line in radiation + ghrelin group (**Figure 1D**).

Discussion

The main finding of the present study was that unfavorable effects of preoperative radiotherapy on colonic anastomosis can be attenuated by ghrelin treatment. In previous studies, it has been shown that growth hormone has beneficial effects on anastomotic healing in rat models [18, 19] and it is known that ghrelin stimulates growth hormone release [20, 21]. On base of that, Ceran *et al*. hypothesized that ghrelin may have positive effects on colonic anastomosis, partly related to increased growth hormone secretion which may promote angiogenesis and consistent with this hypothesis and they found that bursting pressures were effectively increased following ghrelin administration [12]. In the present study, bursting pressure in radiation group was significantly lower than in control group and this decrease has been significantly prevented with ghrelin treatment.

Collagen structural organization is an essential component of colonic anastomoses healing and tissue hydroxyproline level is an important indicator of collagen synthesis [22]. In the present study, hydoxyproline levels were significantly decreased in radiation group and this effect of radiation was prevented by administration of ghrelin. Similarly, Yu *et al*. showed that ghrelin treatment increases hydroxyproline levels in the gastroenterostomy site besides the positive effects of ghrelin on the bursting pressure [13].

TNF-α and IL-1β are cytokines with multiple biological activities including regulation of inflammation and cytotoxic effects [23]. In our study, serum TNF-α and IL-1β levels were increased in radiation group compared to the control group, and decreased significantly after ghrelin treatment. This showed us that activation and infiltration of neutrophils, which play a precipitating role in tissue damage, has been inhibited by melatonin and thus inflammatory response has decreased. Wu et al. have investigated the effects of ghrelin on intestinal ischemia repefusion and found that the elevetion of TNF-α in ischemia-reperfusion group was prevented by 33% [24]. Similarly, Warzecha *et al*. showed that ghrelin decreased IL-1 levels in rats with cysteamine induced duodenal ulcers significantly [25].

Reactive oxygen radicals may arise as a result of inflammation or immune-mediated injury [26, 27] and luminol detects these reactive oxygen radicals such as H_2O_2, OH-, hypochlorite, peroxynitrite and lipid peroxyl radicals, while lucigenin is selective for superoxide radicals [17]. Işeri et al. showed that ghrelin reduced the levels of luminol in rats with alendronate-induced gastric damage [28]. Hedeyati et al. have found that ghrelin may attenuate the homocysteine-induced endothelial dysfunction in porcine coronary arteries and human endothelial cells by preventing the increase in the levels of lucigenin [29]. In the present study, luminol and lucigenin levels were significantly higher in radiotherapy group than in control group and ghrelin treatment was protective against this oxidative stress caused by preoperative radiation therapy.

Kuzu *et al.* have reported that chemotherapy and radiotherapy may impair anastomotic healing [30]. Similar to his findings, in the present study, impaired epithelial structure, intense inflammation and increased synthesis of collagen in the lamina propria were observed in the radiation group. Ghrelin treatment following radiation have minimized the unfavorable effects of radiotherapy and we observed that the muscular structure and collagen synthesis were regular and inflammation was regressed.

In conclusion, exogenous ghrelin is associated with functionally and histopathologically protective effects against injury in the anastomotic site caused by radiation in this rat model. These results need to be verified by appropriate clinical studies.

Disclosure of conflict of interest

None.

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