The Effect of the iNOS Inhibitor S-Methylisothiourea and Hyperbaric Oxygen Treatment on Radiation Colitis in Rats

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Abstract

Introduction : **External radiotherapy is one of the main treatment modalities for a variety of malignancies. However, the lower gastrointestinal tract is sensitive to the ionizing radiation. Hyperbaric oxygen treatment (HOT) has been suggested as a viable treatment for refractory radiation colitis, but the effect of S-Methylisothiourea (SMT) in the radiation colitis have not reported.**

Aim : **To investigate the effect of SMT, HOT and the combination of both in an acute radiation-induced enterocolitis model.**

Methods : **Sixty Sprague-Dawley rats were divided randomly into five equal groups. A single dose of gamma irradiation (25 Gy) was administered through the colorectal region to anesthetized rats. In the control group, we applied 2 ml of saline solution intraperitoneally for five days. In the HOT group, 100-per-cent oxygen at 2.5 atm pressure was applied for five days. In the SMT group, 10 mg/kg/day of SMT was applied intraperitoneally for five days. In the HOT+SMT group, HOT and SMT were both applied in the same dosages as in the preceding two groups. At the end of five days, the rats were sacrificed and colon samples were collected for histological grading. Blood samples were collected to test for : tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), IL-1β, transforming growth factor-β (TGF-β) and intercellular adhesion molecule-1 (ICAM-1) mRNA.**

Results : **The TNF-α, IL-1β, IL-10 and TGF-β levels were reduced by SMT, HOT and HOT+SMT applications ($p < 0.05$). However ICAM-1 mRNA levels were not significantly lower (p:0.19). The microscopic scores differed significantly between the SMT, HOT and HOT+SMT groups and the control group. There was significant improvement histologically, especially in the HOT+SMT group. When we compared the weight of the rats before and after the study, weight loss was significantly lower in the SMT, HOT and HOT+SMT groups compared with the control group ($p < 0.05$).**

Conclusions : **HOT and SMT together were significantly more effective in preventing weight loss and in reducing inflammation and the severity of colitis histology when compared with HOT and SMT separately.** (Acta gastroenterol. belg., **2015**, 78, **8-13**).

Key words : S-Methylisothiourea ; hyperbaric oxygen treatment ; radiation colitis ; inducible nitric oxide synthase (iNOS) inhibitor.

Introduction

External radiotherapy (RT) is one of the main treatment modalities for a variety of malignancies. Many patients with gynecologic, urologic and rectal cancers are treated with RT to the pelvis to deliver x-rays to the target tissue and to limit damage to other organs. However, the lower gastrointestinal tract, including the sigmoid colon and rectum, is very sensitive to the side effects of ionizing radiation and a significant proportion of patients suffer from acute or chronic colitis after pelvic irradiation, despite preventive strategies to limit toxicity (1-3).

Hyperbaric oxygen treatment (HOT) has recently been suggested as a viable treatment option for patients with refractory radiation colitis and proctitis (4,5). However, 35 to 50 per cent of patients have not shown a partial or good response to the treatment and there has been a low complete-cure rate (6,7). In a randomized, controlled, crossover study, the absolute risk reduction was 32 per cent with HOT and the number needed to treat was three (8). These studies showed that, despite the promising results of HOT, new therapeutic approaches are still needed for a significant group of patients.

S-Methylisothiourea (SMT) is a potent and selective inhibitor of inducible nitric oxide synthase (iNOS) (9). It suppresses oxidative stress significantly and demonstrates anti- inflammatory properties (10). SMT has been shown to be a greater inhibitor of iNOS in immunostimulated, cultured macrophages than other NOS inhibitors (9). Ionizing radiation induces iNOS-mediated epithelial secretory dysfunction in intestinal cells (11). The protective effect of SMT in nitrogen mustard-induced lung toxicity and acetaminophen-induced hepatotoxicity has been shown in rat models (10,12). It has also been potentially protective in intestinal ischemia and reperfusion injury in rats (13). The use of SMT hemisulfate improved the healing of colonic anastomosis in rats by accelerating the proliferative stage of healing (14).

SMT, as a strong iNOS inhibitor with antioxidant properties, may have a useful role in the treatment of radiation colitis. The present study aimed to investigate the efficacy of SMT alone and in combination with HOT in an experimental acute radiation-induced enterocolitis model in rats.

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Methods

Animals and Study Design

A total of 60 female Sprague-Dawley rats weighing 200 to 300 g were procured for the study from the Department of experimental animals of the Gulhane Military Medical Academy. The rats were housed in a climate-controlled animal care facility with standard rat feed and tap water at room temperature. All rats were kept according to the standards of the Department of Experimental Animals (temperature : 20-25°C, humidity : 70-80%, 12-h/12-h light/dark cycle). All animals received humane care in compliance with the institution's guidelines, as outlined in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health. The Animal Ethics Subcommittee of the Gulhane Military Medical Academy approved the study.

The animals were placed randomly in five groups containing 12 rats each (Fig. 1). They were not feed but continued to drink for 24 hours before the study for colonic preparation. All rats were weighed before and after the study. Radiation colitis was induced in Control group, SMT only group, HOT only group, and SMT+HOT group. Rats in sham group were provided same conditions, but not administered gamma irradiation. Theratron 780E Cobalt-60 Teletherapy Unit (AECL Medical) available in the Radiation Oncology department was used for gamma-irradiation of the rats. All rats were anesthetized with an intraperitoneal injection of 50 mg/kg of ketamine hydrochloride and 10 mg/kg of Xylazine, and then fixed onto the treatment couch in supine position for single-fraction gamma-irradiation. Radiation was delivered through an anterior 3 × 4 cm single portal focused on the colorectal region with a source to skin distance of 80 cm. Sparing of extremities, head, thorax, and extremities during gamma-irradiation was assured by lead shielding. All rats were irradiated individually using a single dose of 25 Gy, which has been shown to be in the dose range for enterocolitis induction (15,16). Following gamma-irradiation, rats were observed until recovery from the anesthetic.

After irradiation, each animal was monitored daily for changes in weight and in the appearance of the anal region. The control group was administered a 2 ml saline solution intraperitoneally for five days. The HOT group received 100-per-cent oxygen at 2.5 absolute atmospheres (ATA) pressure. HOT was administered immediately using a special animal hyperbaric chamber (made in Etimesgut Military Equipment Factory ; Ankara, Turkey) after radiation exposure for 90 min. The chamber was flushed with 100% oxygen at a rate of 5 L/min to avoid carbon dioxide accumulation. Compression and decompression of the chamber was completed gradually in 5 min. All administrations were started to prevent biological rhythm changes at the same time every morning for five days. The temperature of the pressure chamber was maintained between 22-25°C (17). The SMT group received 10 mg/kg pf SMT intraperitoneally, both for five days. The combination group received both treatments at the same time for five days. The sham group did not receive any treatment during the study. At the end of the study, all the animals were euthanized by overdose of Ketamine/Xylazine anesthesia. Colon samples, 10 cm from the distal part of the colon, were collected for histopathological grading. Blood was collected from heart puncture and allowed to clot for 30 min at 37°C temperature, for estimation of several parameters in serum. Serums were centrifuged for 10 min at 3,000 rpm at 4°C. The supernatants were preserved at -80°C until assayed.

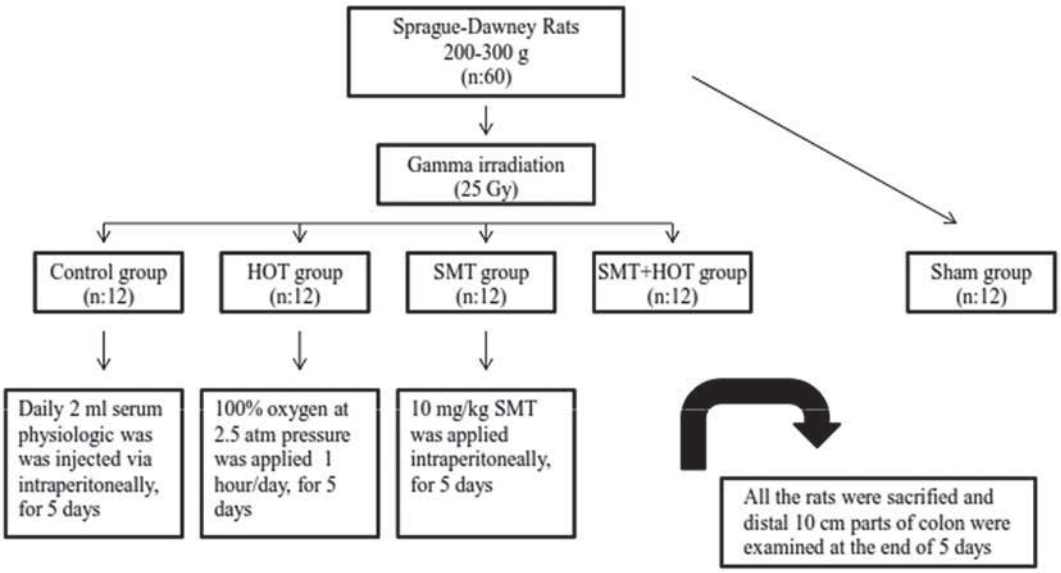

Fig. 1.

Table 1. — **Criteria for microscopic scoring of colonic lesions**

Inflammatory cell infiltration *(lymphocytic)*	0 (none), 1 (moderate), 2 (severe)
Cyrpt Distortion Loss of Goblet Cell	0 (none) 1 (mild) 2 (moderate) 3 (severe)
Submucosal edema Inflammatory cell infiltration (PMNL) Ulceration Crypt Hyperplasia	0 (none) 1 (localized) 2 (mild) 3 (moderate) 4 (severe)

Histologic scoring system with small modifications of Gue *et al*.
PMNL, polymorphonuclear leukocyte.

Histopathological and Biochemical analyses

All materials were fixed in a 10-per-cent neutral-buffered formalin for at least 72 hours and then sampled. After routine overnight tissue processing, they were embedded in paraffin. Standard four-micrometer-thick sections were prepared from each tissue block. Following deparaffinization and rehydration, sections were stained with hematoxylin and eosin. Tissue sections examined under an Olympus BH 2 photomicroscope (Olympus, Tokyo, Japan). A pathologist, who worked on a double-blind basis from the beginning of the study, evaluated all the tissue samples and scored them semi-quantitatively by the presence and degree of edema, inflammatory cell infiltration, ulceration, crypt distortion and hyperplasia as well as goblet cell loss. Histologic scoring was performed by the previous described criteria of Gue M *et al*. with small modifications (18) (Table 1).

TNF-α, IL-1β, IL-10, TGF-β and ICAM-1 mRNA levels were measured by an ELISA (enzyme linked immunosorbent assay) method in blood samples at the end of the study. All samples were assayed in triplicates using the commercial kits according to the manufacturer's instructions. (ICAM-1 : R&D Systems, Inc, USA. TNF-α, IL-1β, IL-10, TGF-β : Invitrogen, CA, USA) and the values were expressed as pg/mL.

Statistical Analyses

All data were analyzed with SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). The results were expressed as median figures. The Kolmogorov-Smirnov test was used to determine the distribution characteristics of continuous variables. The normally distributed variables were compared with one-way ANOVA for multiple group comparison and a Tukey's post hoc test. The Kruskal-Wallis test was used for multiple-group comparison of variables without normal distribution, and a Bonferroni-adjusted Mann-Whitney U test was used for post hoc analysis. A p-value of less than 0.01 was considered statistically significant for overall group analyses, and a p-value of less than 0.05 was considered statistically significant for dual comparisons.

Results

Weight of rats

We took the weights of the rats before the study : sham group 214.1 ± 6.7 g, control group 215.7 ± 8.3 g, SMT group 216.4 ± 7.8 g, HOT group 212.9 ± 7.7 g and SMT+HOT group 213.5 ± 8.6 g). We then took their weights after the study : sham group 213.8 ± 6.5 g, control group 195.4 ± 7.5 g, SMT group 204.5 ± 7.6 g, HOT group 202.6 ± 7.6 g and SMT+HOT group 205.5 ± 8.7 g). Weight loss was notably lower in the SMT, HOT and SMT+HOT groups compared with the control group ($P < 0.05$).

Biochemical assessments

ICAM 1 levels in the sham group was higher than the HOT group, but no significant difference was found between other groups in terms of ICAM 1 levels. While TNF α in the control group was highest compared to other groups ($P < 0.05$), there was no different among SMT, HOT, and SMT+HOT groups ($P > 0.05$). TNF α levels were higher in the sham group than the SMT+HOT group ($P = 0.048$). IL-1β levels in the control group were higher compared to SMT, HOT, and SMT+HOT groups ($P < 0.05$), but no significant difference were existed between control and sham groups ($P > 0.05$). Moreover, IL-1β in the sham group was higher than in the SMT and SMT+HOT groups, whereas there was no different among SMT, HOT, and SMT+HOT groups in IL-1β levels. Although TGF-β levels in the control group were increased compared to sham, SMT, and SMT+HOT ($P < 0.05$), no significant difference was existed all of the comparisons for TGF-β level ($P > 0.05$). IL 10 levels in the control group were significantly higher compared to others groups ($P < 0.001$), but no significant difference was found among the remaining four groups ($P > 0.05$) (Table 2).

Histopathological assessments

In histopathological analysis, no pathologic findings were observed in the intestinal mucosa of the sham

Table 2. — **Biochemical and histopathological results**

Variables	Group A (Control, n = 12)	Group B (SMT, n = 12)	Group C (HOT, n = 12)	Group D (HOT+SMT, n = 12	Group E (Sham, n = 12)	P value
Inflammatory parameters						
TNF α (pg/mL)	21.5 ± 5.3	14.3 ± 4.1	13.7 ± 2.2	10.2 ± 3.7	15.1 ± 3.1	< 0.001
IL 1β (pg/mL)	105.4 ± 37.9	41.5 ± 16	62.1 ± 15	39.6 ± 16.4	82.8 ± 26	< 0.001
IL 10 (pg/mL)	33.9 ± 5.9	23.9 ± 4.9	22.8 ± 4.8	21.1 ± 1.9	19,8 ± 2	< 0.001
TGF β (pg/mL)	68.5 ± 32.1	20.4 ± 7.8	41.5 ± 24.1	27.4 ± 5.6	33.1 ± 42.7	0.001
ICAM 1 (pg/mL)	267.7 ± 46.3	273.5 ± 45.1	236.9 ± 36	260.3 ± 39.6	302.5 ± 39.8	0.019
Histopathological scores	17.3 ± 1.6	11.1 ± 1.2	10.2 ± 0.9	7.41 ± 0.5	1.2 ± 0.1	< 0.001

SMT, S-Methylisothiourea ; HOT, hyperbaric oxygen treatment ; TNF α, tumor necrosis factor alpha ; IL 1, interleukin 1 ; IL 10, interleukin 10 ; TGF β, transforming growth factor beta ; ICAM 1, intercellular adhesion molecule-1.

Significant Tukey post-hoc comparisons for TNF α ; P = 0.001 for A *vs*. B, A *vs*. C, and A *vs*. D, P = 0.003 for A *vs*. E, P = 0.048 for group D *vs*. E.

Significant Tukey post-hoc comparisons for IL 1β ; P < 0.001 for A *vs*. B and A *vs*. D, P = 0.002 for A *vs*. C, P = 0.003 for E *vs*. B, P = 0.001 for E *vs*. D.

Significant Tukey post-hoc comparisons for IL 10 ; P < 0.001 for A *vs*. B, A *vs*. C, A *vs*. D and A *vs*. E.

Significant Tukey post-hoc comparisons for TGF β ; P = 0.001 for A *vs*. B, P = 0.006 for A *vs*. D, P = 0.023 for A *vs*. E. Significant Tukey post-hoc comparisons for ICAM 1 ; P = 0.009 for C *vs*. E.

Significant Tukey post-hoc comparisons for histologic scores ; P < 0.001 for A *vs*. B, A *vs*. C, A *vs*. D, A *vs*. E, B *vs* E, C vs E, and D *vs* E, P = 0.009 for D *vs* B, P = 0.023 for D *vs* C.

group. Radiation induced intestinal tissue damage was the most severe in the control group and histologic score was highest in the control group (P < 0.05). The improvement in histopathological findings was considerable in the HOT+SMT group when compared to SMT and HOT groups (P < 0.05). The severity of the radiation colitis in the HOT group was milder than the SMT group, but no significant difference was existed between two groups (Table 2) (Fig. 2).

Conclusions

Radiation therapy is increasingly utilized in the management of several tumors. The reported rates of acute and late gastrointestinal toxicity differ among studies ; however, a vast majority of patients undergoing radiotherapy to the abdominal or pelvic region suffer from gastrointestinal symptoms with varying degrees of severity which may profoundly affect their quality of life (19). Besides being a dose-limiting factor, radiation-induced toxicity may be life-threatening in its most severe form. Patients experiencing acute toxicity during radiotherapy may be at higher risk for late morbidity (20). Thus, the management and prevention of radiation- induced acute bowel toxicity merits special attention. We hypothesized that SMT, as a potent iNOS inhibitor with antioxidant properties, could prevent or treat radiation-induced acute colitis.

Our study demonstrated that combining HOT and SMT treatments resulted in considerably better histopathological, laboratory and clinical results when compared to using the individual treatments alone.

Radiation induces the expression of cytokines such as TNF-α, IL-1β, IL-10 and TGF-β (21). These cytokines induce endothelial cells, bringing about leukocyte adhesion, macrophage activation and extravasation (22). The number of adherent leukocytes increases notably a few hours after irradiation (23). Vascular cell adhesion molecules and ICAMs are important for adhering and transmigration phases (24). ICAM-1 expression on the endothelium plays a critical role in the migration of activated leukocytes into the areas of inflammation (25). The increased expression of ICAM-1 in the colon of patients with inflammatory bowel disease has been reported (26). Some studies have shown that ICAM-1 expression can be induced by ionizing radiation (27). Moreover, ICAM-1 knockout rats had no increase in inflammatory cell infiltration into the lung in response to irradiation (28). ICAM-1 may play an important role in causing indirect tissue destruction mediated by leukocyte infiltration in radiation-induced colitis.

NO is a free radical molecule which is produced by the reaction of NOS. Macrophage oxidation of L-arginine to NO is catalyzed by NOS. It is generally recognized that NO plays a crucial role in inflammation. The metabolism of NO has been commonly investigated in wound healing, particularly in intestinal anastomosis. NO is an important cell mediator of tissue repair. The release of NO is found to escalate during trauma, sepsis and surgical stress. There are three enzyme isoforms : neuronal, endothelial and inducible (29).

SMT is a potent and selective inhibitor of iNOS (9). It suppresses oxidative stress significantly and shows anti-inflammatory properties (10). SMT has been shown to be a greater inhibitor of iNOS in immunostimulated, cultured macrophages than other NOS inhibitors (9). In experimental colitis models, the formation of colonic NO is increased in association with the advance of colitis (30).

HOT is generally used as a supplementary treatment for miscellaneous inflammatory diseases (31-33). HOT therapy can be regarded as a treatment alternative after

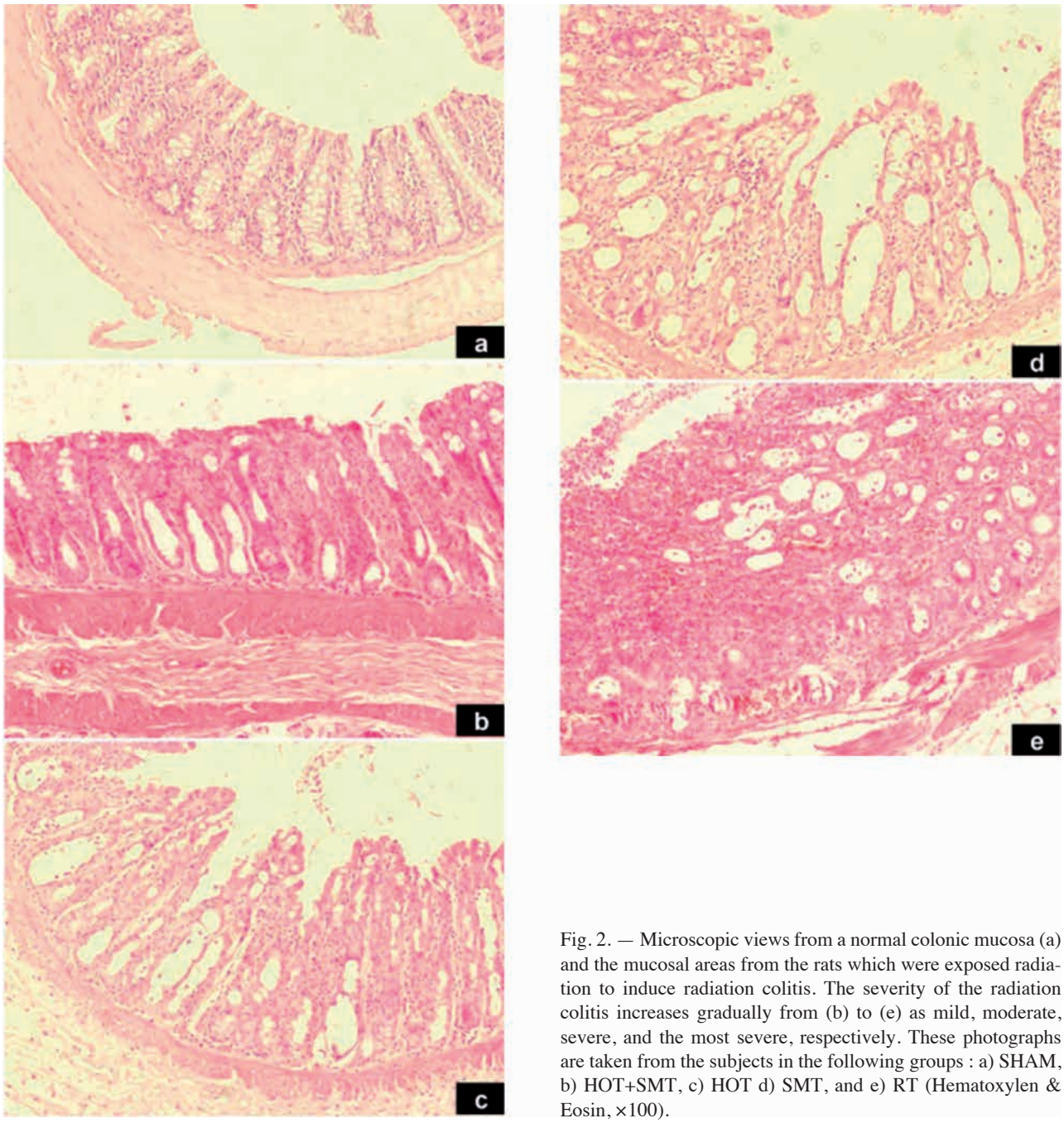

Fig. 2. — Microscopic views from a normal colonic mucosa (a) and the mucosal areas from the rats which were exposed radiation to induce radiation colitis. The severity of the radiation colitis increases gradually from (b) to (e) as mild, moderate, severe, and the most severe, respectively. These photographs are taken from the subjects in the following groups : a) SHAM, b) HOT+SMT, c) HOT d) SMT, and e) RT (Hematoxylen & Eosin, ×100).

the failure of standard treatments in patients with radiation colitis (34). The explanation for the use of hyperbaric oxygen is its ability to increase the oxygen gradient in hypoxic tissue by inducing the formation of new blood vessels. New angiogenesis stimulates the blood supply and decreases the ischemia and necrosis. The effect of HOT therapy has been investigated in many studies, such as those into experimental colitis (35). Gouello *et al*. showed that HOT treatment provided clinical alleviation and may prove to be an effective treatment alternative in patients with radiation colitis (36). Another study showed the beneficial effects of combining HOT therapy with aminoguanidine, a selective iNOS inhibitor, in experimental colitis in rats (37).

To the best of our knowledge, ours is the first study investigating the use of SMT alone and in combination with HOT for experimental acute radiation-induced enterocolitis. In this study TNF-α, IL-1β, IL-10 and TGF-β levels were reduced by SMT, HOT and HOT+SMT applications ($p < 0.05$). However, ICAM-1 mRNA levels were not significantly lower among the groups (p:0.19). The microscopic scores differed significantly between SMT, HOT, HOT+SMT groups and the control group. There was significant improvement histologically,

especially in the HOT+SMT group. When we compared the weight of the rats before and after the study, weight loss was significantly lower in the SMT, HOT and HOT+SMT groups compared with the control group ($p < 0.05$).

The combined use of HOT and SMT was significantly effective in preventing weight loss and in reducing the inflammatory activities and severity of colitis histology compared to HOT and SMT separately. Future studies are needed to address the combined effect of SMT and HOT in radiation colitis.

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