IRRADIATION AND HEALING OF COLONIC ANASTOMOSES

J. Biert

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an experimental study in the rat

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Jan Biert

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Promotor:	Prof. dr. Th. Wobbes
Co-promotores:	Dr. Th. Hendriks
	Dr. J. Hoogenhout

Manuscript commissie:	Prof. dr. A. van der Kogel
	Prof. dr. P. de Mulder
	Prof. dr. P. van den Broek

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Chapter 1

INTRODUCTION, REVIEW OF THE LITERATURE AND AIM OF THE STUDIES

1. ANASTOMOTIC HEALING OF THE COLON AND RECTUM

In clinical practice undisturbed colorectal anastomotic healing takes about seven to ten days. After this period the strength of the anastomosis has reached normal values, or is even greater than that of the surrounding tissue. The condition of the patient changes from catabolic to anabolic, and he is able to eat and have normal defaecation, indicating that intestinal passage has been restored. The laparotomy wound heals progressively, and although the microscopical process of intestinal wound healing will continue for the next months, the surgeon considers the patient healed.

There is a vast amount of literature concerning anastomotic healing of the colon and rectum. This is the result of the high percentage of healing disturbances that are encountered in clinical practice. The effects of this impaired healing are a severe threat for the life and well-being of the patient, especially if it concerns low colorectal anastomoses. Leakage of the large bowel anastomosis increases the mortality rate from 7 to 22% (1).

Otherwise, morbidity is also increased: abdominal abcesses, sepsis, Multi Organ Dysfunction Syndrome (MODS), and various other surgical and medical complications may follow. A leaking colorectal anastomosis often necessitates a relaparotomy; usually the anastomosis is dismantled with construction of an (often definitive) diverting ileostomy or colostomy. After this, a number of reoperations for septic complications can be expected; this situation leads to an increase in stay on the intensive care, hospital stay and overall costs.

Anastomotic leakage rates vary in different reports from 1 to 51% (1-5), and a mean leakage rate of approximately 13% can probably be considered as "normal" (1). There are many factors contributing to this difference. At first one has to consider the way in which the leakage of the anastomosis is diagnosed. If a routine bowel X-ray is made after construction of the anastomosis minimal leakage is a phenomenon reported in 24 % of patients (4,6), while half of these leakages have no clinical impact, and would otherwise not have been diagnosed.

While anastomotic leakage is considered to be the ultimate form of a healing disturbance, other forms of impaired healing can be distinguished as well, like a delay in formation of anastomotic strength, formation of a stenosis or a fistula, and bowel wall necrosis. Many factors responsible for impaired

healing, and countermeasures have been studied both clinically and experimentally, and are well-known:

1. The suture technique of the surgeon plays an important role (7,8).
2. There are technical factors, which have their effect on the blood (and oxygen) supply of the anastomosis: a) insufficient mobilisation of the proximal colon leads to a distraction force on the anastomosis, b) increased marginal dissection leads to impaired vascularisation of the anastomotic limbs, and c) haematoma in the anastomosis may compromise the vascularization of the anastomotic area as well. Incomplete approximation of bowel ends (in circular stapled anastomoses this is shown by incomplete "doughnuts") may lead to a higher chance of leakage if no additional sutures are used (9), and often a protective, proximal fecal diversion is added in these situations with subsequent increased morbidity. Of course, surgical skill in respect to these technical factors is very important, and this leads to the appreciation of the surgeon to be the most important single factor concerning anastomotic integrity (1).
3. Medical situations influence anastomotic repair: the use of drugs like cytostatic agents (10-12), and chronically used steroids (13,14) lead to disturbed healing. Other important medical conditions are diabetes mellitus (15) and extreme malnutrition (16,17). An increase of age is associated with higher leakage rates by some (18), although this has not been confirmed in a study in our own laboratory (19).
4. Most surgeons will consider a local or generalized peritonitis to be a contra-indication for the construction of bowel anastomoses (18,20).
5. Proper bowel lavage prior to the resection decreases the chance of faecal soiling during the operation, and also decreases the faecal flow through the anastomosis in the first 24 hours (18). This beneficial effect has been denied by Burke *et al.* who found no beneficial effects of bowel lavage on anastomotic failure in a group of 186 patients (21).
6. The additional use of systemic and local antibiotics has been proven to be effective in preventing leakage (22).
7. Irradiation has always been considered to be detrimental for anastomotic healing in clinical practice (18,23,24). This has been

confirmed experimentally: Morgenstern found 66% colonic anastomotic leakage in dogs after fractionated irradiation with 8 x 5 Gy in 4 weeks (25). Ormiston also reported increased anastomotic breakdown after irradiation with 15 or 20 Gy (single dose) of the ileum in rats (26), and Degges found decreased anastomotic wound strength 5 days after 14.5 Gy (single dose) preoperative irradiation and colonic resection in rats (27). Others had similar results in rats or dogs (28-34). On the contrary, there have also been reports of unhampered experimental anastomotic healing following preoperative irradiation (35,36). Total dose, fractionation, dose rate, irradiated volume, time sequence of irradiation and surgery, and surgical technique, all seem to be of importance (37,38).

8. Many surgeons believe that the construction of a diverting colostomy or ileostomy proximal to the anastomosis may create better conditions for anastomotic healing. This idea neglects the report that the mucosal epithelial cells derive their nutrition (butyrate and other short-chain fatty acids) from the faeces (39); a proximal decompression does not protect against anastomotic disruption, both clinically (18) and experimentally (39-42).

Currently, there exists a lot of research interest in delineating the hazards for anastomotic repair under various conditions, and in improving healing under suboptimal conditions.

2. SOME SURGICAL ASPECTS CONCERNING COLORECTAL CANCER TREATMENT

Surgery is considered to be the primary and most important way of treatment of patients with colorectal cancer. Evolution of surgical techniques, instrumentation and a better medical support (intensive care facilities, and the introduction of new, powerful antibiotics (43-48)) have only led to slightly better prognosis or improved quality of life. Survival of the patient is not the only goal in the treatment of colorectal cancer; the quality of life has to be taken in consideration also. For example, local recurrence is a disaster for the patient with only limited treatment options. In this scope it is important to define whether there is a local recurrence

after an intentionally curative resection, or after a palliative resection (49). Some aspects of surgical treatment have changed in recent years. Technical innovations, like stapling devices, enable the surgeon to prevent the construction of a proximal diverting colostomy, by the construction of low or very low colorectal anastomoses using the double stapling technique. This has created an alternative to most abdomino-perineal resections (4,6,9,50).

General ideas about the appropriate length of the disease-free margin at the resection site, have evolved. A free margin of 5 cm has long been considered to be essential; this however depends on the time and method of measurement: in situ the margin may be 5 cm, but after resection, in the pathological laboratory this may have shrunk to 2.5 cm (51). Nowadays a 1 cm margin is considered to be sufficient (52,53), and more attention is focussed on lateral margins because of lymphatic spread of tumor cells (52,54,55). This is clearing the way for more low anterior resections instead of abdomino-perineal resections. Of course the prevention of a colostomy after a low anterior resection is considered to be a major contribution to the quality of life of the patient.

The attention to the lateral margins has led to a new dissecting technique, total mesorectal excision (TME), with an extensive perirectal lymph-adenectomy which may further improve local control and survival (55-60). All the surrounding perirectal fat is dissected carefully up to the sympathic nerve plexus or beyond, and this is continued until the pelvic floor is reached. Trials using these techniques are still in progress.

Another new technique is the laparoscopic or laparoscopy-assisted colectomy or rectosigmoidectomy (61,62). This technique however is still experimental: long-term results are not yet available, and there have been alarming reports of tumor spread in portal canal sites (63).

Furthermore, in the medically unfit patients, local excision of rectal tumors has been advocated (64).

Hard to measure, but of vital importance for the overall treatment result is surgical skill. Peroperative tumor perforation has been proven to result in a higher incidence of local recurrence (65).

Surgery as a single treatment modality can not cure all patients. Considerable mortality and morbidity must be expected because of local recurrence or distant metastasis, and this has led to the development of

adjuvant therapies. Most frequently used treatment modalities are irradiation, cytostatic agents and combinations of these. A new treatment modality increasingly used is hyperthermia. In the next sections various aspects concerning radiation therapy will be discussed.

3. TECHNICAL ASPECTS OF RADIOTHERAPY

Time sequence, methods of application

Radiation therapy is considered to be an useful adjuvant therapy to surgery with the aim of obtaining downstaging of the tumor before resection, and better local control after resection. This additional treatment can be given in various ways: preoperatively, intra-operatively, postoperatively, intra-cavitarily (intra-rectally) and combinations of these. Clinical and experimental aspects will be discussed in the next section.

In colorectal irradiation, both clinically and experimentally, photon beams are frequently used, and - less frequently - electron beams (in intra-operative radiotherapy and experimentally); effectiveness depends on the total dose, number of fractions, fraction dose, dose rate, irradiated volume, number of portals, time sequence in relation to surgery, tumor characteristics, and other factors (66).

Dose fractionation, time and volume effects

In clinical practice, fractionation of the total radiation dose is commonly used (67). The beneficial effects of fractionated irradiation are the result of different radiobiological behaviour of tumor cells and normal tissues (66,68-70). In this way, several advantages can be obtained. First, the use of fractionation permits a higher total dose to be used. Normal cells may recover from earlier radiation damage, probably better than tumor cells (67). Secondly, the extension of the treatment in time allows for reoxygenation of hypoxic tumor cells; since hypoxic cells are less sensitive to irradiation, reoxygenation will increase the sensitivity of the tumor (67). By using daily fractions of 2 Gy (usually 5 days a week) it is possible to gain good local control with few late side effects (71,72). The importance of the use of a fractionation regime has been emphasized before (73). Of course, the total dose used is dependent on clinical experience, histology of the tumor, extent

of the target volume and way of application of the radiation dose. The use of multiple beams will lead to a larger irradiated volume, however, permitting a higher local dose on the target area with less side effects on the surrounding normal tissues (68,74).

Radiation sensitizers

Because side effects of radiation therapy on normal tissues limit the total dose applied, studies are done to increase the radiation effect without increasing the total dose.

Several chemicals modify the radiosensitivity of tissues. One of the most powerful radiosensitizers is oxygen. Although not completely understood, its mechanism is thought to be a fixation of radiolesions, otherwise considered repairable (66). Other chemicals capable of radioenhancement are those with a high oxydizing potential, and some chemotherapeutics (66,75). The effect of these chemotherapeutics may result in a synchronisation of surviving cycling cells, leading to tumor cells which enter radiosensitive phases in the cell cycle and become susceptible for a subsequent radiation dose. This is achieved by effects on DNA structures, inhibition of repair of sublethal damage, inhibition of DNA synthesis and modification of DNA structures by incorporation of halogenated pyrimidines (66).

Radiation protectors

Side effects of radiation therapy can be very hazardous. For example, the application of radiation therapy has long been considered detrimental for bowel anastomotic repair, both clinically and experimentally (18,25,26,30,76-78). The morbidity, associated with side effects, has encouraged the use of radioprotective agents aimed at reducing the injury. The application of vitamin A (77,79), a sulfhydryl radioprotector called WR-2721 (76,80), elementary diet (81) or sodium meclofenamate (79) can prevent colonic injury after pelvic irradiation. However, it remains unclear if the radiation effects on tumor cells are reduced as well.

If intra-operative or postoperative irradiation are planned, other (surgical) options provide protection of normal tissues from radiation side effects: the use of omentum slings or omentum pedicle flaps, pelvic floor reconstruction with residual peritoneum, temporary balloon devices or spacers, a distended

urine bladder (82), and -in case of intra-operative radiation therapy (IORT)- lead shielding (83).

Combined treatment modalities

There are several ways to enhance the combined effect of surgery and radiotherapy. The goals of these additional adjuvant treatment modalities are: better local control, less distant metastasis, a longer disease-free interval and improved survival.

- The use of chemotherapy has been examined extensively in colorectal cancer patients (84-88). 5-Fluorouracil is always used, mostly in combination with levamisole or leucovorin.
- The application of hyperthermia to the irradiated area can be used to enhance the effectiveness of surgery and radiotherapy. Hypoxic cells are relatively radioresistant, but very sensitive to heat. Since, in hypoxic areas, blood flow is reduced, heat can not be disposed of as quickly as in normal tissues, with subsequently increased cell kill. This effect occurs at temperatures of 42°C and above. The rise in temperature modifies the structure of lipoproteins composing the cell membrane, and causes denaturation of thermolabile proteins. This affects the membrane and enzymatic functions. This effect is dose related in both temperature and application time (66). Furthermore, heat leads to hyperthermic radiosensitation by a quantitative response (89). Thus, the application of heat and radiation therapy can have a additive or even synergistic effect on tumor cell kill. Still, the combination of radiation therapy, hyperthermia and surgery has not (yet) been used often in colorectal cancer.
- Of course, the additional effects of chemotherapy and hyperthermia can be combined, leading to a very intensive treatment. This combination of irradiation, hyperthermia, chemotherapy and surgery has been used incidentally in patients with advanced or recurrent cancer, and is still experimental (90).

4. CLINICAL ASPECTS OF SURGERY AND IRRADIATION OF COLON AND RECTUM

The effectiveness of high dose radiotherapy alone on human colorectal

cancer has been proven in a study on patients, unfit for, or refusing surgery (91). Here, doses up to 60 Gy in 30 fractions of 2 Gy in 6 weeks led to complete tumor regression after irradiation in 50% of patients with mobile tumors, with a 5 year survival rate of 48% (91).

There has been a lot of discussion about the place of irradiation as an adjuvant therapy in colorectal cancer surgery (73). Many of the trials in the past have been retrospective. Those which are prospective tend to lack consistency concerning important aspects of the trial concept, like surgical technique. Since clinical trials have to be large enough to get statistical significance it is neccessary to include more patients and hence, more hospitals in the study. This has its influence on the study's consistency.

In the past, postoperative irradiation has been used frequently (65,92). The advantage is that a histological staging of the disease is present, that there is information on the surgical clearance and tumor-free margins, and in the event of a low anterior resection it can be applied safely after healing of the anastomosis. There are also disadvantages. During laparotomy the rectoperitoneal fold is incised. Postoperatively, the small bowel may become fixed in the true pelvis by adhesions. Since the small bowel is very susceptible for ionizing radiation (68), side effects may evolve. The total wound volume is large, and this demands for an even larger irradiated volume. The surgical operation area is relatively hypoxic, and spilled or residual tumor cells situated here will be relatively radiation resistent (66). Finally both afferent and efferent bowel limbs of the anastomosis are irradiated; this can only increase the already high incidence of post-irradiation stenosis (37,71). This was shown in a study where postoperative irradiation led to an increased cumulative rate of late bowel obstruction with a possible lower 5-year survival (92). Other studies have demonstrated a positive effect of postoperative irradiation on local control in selected cases (93). The combination of local excision of rectal tumors with postoperative irradiation, in patients with localised disease, was proven to be an alternative to extensive surgery (94).

Nowadays, more attention is focussed to preoperative irradiation, which is considered to be a promising adjuvant treatment to surgery (65,92,95-107), with advantages over postoperative irradiation (65,92,108,109). Increased local control has been demonstrated in several studies (65,95-97,102-107), as well as prolonged survival (95,102-104); in other studies there was no

difference in survival rate (65,96,105-107). However, treatment protocols were not similar, and these differences can have an enormous impact on the eventual irradiation effects; the most promising prospective trials that advocate the use of preoperative radiotherapy are lacking a standardized way of surgery (98,100). A dose related response can be observed (105). If the tumor has extrarectal fixation, preoperative irradiation with a dose of 45 Gy in 25 fractions of 1.8 Gy can downstage the tumor by cytoreduction, allowing for long-term local control and sphincter saving (99,103).
Anastomotic complications are not unfrequent after preoperative irradiation and subsequent surgery, and percentages vary up to 28% after low anterior resections (100,105). Others report that irradiation results in normal rates of anastomotic leaks (2,101,110). Patient selection and surgical skill seem to be important factors in these studies.
In one of the studies preoperative irradiation with 25 Gy in 5 fractions, followed within 1 week by rectal resection, was not complicated with an increased incidence of anastomotic dehiscence (98). In another study 40 Gy of preoperative irradiation, in fractions of 1.8-2.5 Gy, was followed by anastomotic complications in 28% of all low anterior resections (100); this was confirmed in another multicenter study (101). Others reported that short-term, preoperative irradiation with 25.5 Gy (fraction dose 5.1 Gy) in one week decreased the incidence of local recurrence relative to postoperative irradiation with 60 Gy, without increased late morbidity as compared to surgery alone after a follow-up of 5-10 years (92).
Another method, which has not gained wide-spread application, is intra-operative radiation therapy (IORT). The surgical wound at the site of a questionable tumor clearance is irradiated, while the surrounding normal tissues are shielded. This technique allows for a large dose on the target area with a small irradiated volume (83,111-116). Residual tumor cells will be attacked directly, leading to a higher probability of local control. The total dose of this treatment is usually low, so that a combination with pre- or postoperative irradiation is needed to increase the total effective dose. For IORT specially equipped operating theaters are neccessary, which have the possibility to allow a radiation dose to be applied to an unresectable place of the operation area, often deep down the true pelvis, while the surrounding structures are shielded to prevent unwanted side effects. Another possibility is to transfer the anaesthetized patient to the department of radiotherapy. Of

course this has many objections, mostly because of operative sterility and logistic reasons. Since this type of irradiation is technically rather complex, and requires an adequate organisation, clinical experience still is restricted and in part experimental; most often only patients with advanced tumors are treated (83,111-115).
Other possibile radiation treatment modalities are "sandwich"-irradiation (a combination of pre- and postoperative irradiation) (97,117), endocavitary irradiation (30,118), and other combinations (82).
The use of radiation therapy as an adjunct to surgery remains the subject of clinical multicenter trials.

5. SIDE EFFECTS OF IRRADIATION ON NORMAL INTESTINE TISSUE

The occurrence of side effects due to radiotherapy can be observed in two periods. First there are the acute side effects, occurring within weeks after surgery as a result of radiation enteritis, with periods of bleeding or anastomotic dehiscence; an inflammatory reaction can be found (118). After a relatively high single dose, microscopically, there is a stop in mitotic activity of the mucosal stem cells within 24 hours after irradiation (66). The height of the villi is diminished; this process continues until the fourth day. After this a gradual recovery takes place, although at a lower speed rate than in normal cell replacement (66,120-124). The number of surviving stem cells per crypt depends on the radiation dose. After single radiation doses higher than 10 Gy, regeneration takes place by horizontal fission of surviving crypts (66). A high dose results in mucosal ulceration after 4 to 5 days; as a result the effects of early radiation enteritis become apparent: diarrhoea, anorexia and infection (66,122). After a low radiation dose of 6 Gy, cell function, as measured by amino acid uptake, rapidly returns to normal; this is however an adaptive mechanism and not simply "healing" (125). Fractionation of the radiation dose results in a higher probability of succesfull regeneration, repopulation, and restoration in the colonic mucosa (69).
Late side effects appear months to years after surgery, even many years after irradiation (126). In these patients early side effects were not always present,

although a severe early reaction is also predictive for late morbidity (127). The risk of both early and late intestinal damage is increased if bowel loops are fixed in the abdominal cavity, as a result of intestinal adhaesions after earlier surgery (82). Irradiation results in thickening of the intestinal wall; microscopically this is the result of edema and intestinal fibrosis (76). Stenosis of the lumen is commonly seen, and sometimes there is superficial ulceration (77,128,129). A microscopic evaluation of the damaged intestinal wall reveals microangiopathy with endoarteritis (128). It has been shown that radiation therapy produces an early and persistant reduction in colorectal anastomotic blood flow (38).

The overall clinical picture of late radiation enteritis resembles the result of intestinal stenosis in inflammatory bowel disease: abdominal cramps, acute and subacute obstructions, perforations and fistulae, and the construction of anastomoses in these areas is often complicated by anastomotic leakage (23,130). Of course the total dose and the size of the irradiated volume is critical for the total of complaints (129,131).

There is a distinct difference between the radiosensitivity of small bowel and the colon/rectum: the small bowel appears to be much more sensitive to irradiation (23,130).

Anastomotic healing of bowel which has been irradiated years before, is frequently complicated by anastomotic leakage, as has been shown in ileoileal anastomoses (23,24). Operations in patients with late radiation-induced damage to intestine tissue, like proctocolitis, strictures, ulceration fistula or spontanaeous necrosis (128), will give rise to more sequelae than operations performed in an early stage, and this asks for a special surgical approach (23,126).

6. AIM OF THE PRESENT STUDIES

Many clinical studies have been undertaken to investigate the possible advantageous effect of radiotherapy as an adjunct to surgery for patients with colorectal cancer. However, consensus seems to be lacking about the potential hazards of irradiation for the healing intestinal anastomosis. Animal studies, although in general difficult to compare because of a wide variation in experimental conditions, have yielded conflicting data in this

respect. Within our laboratory, much experience exists with the assessment of anastomotic healing in rats (11,12,15,19,132-135). These animals are considered to be suitable for irradiation experiments (136). The present series of studies was designed to answer the following questions:

- Is irradiation of the intestine, followed by anastomotic construction using irradiated tissue, by definition detrimental to early wound repair?

- What is the effect of a combination of preoperative irradiation and other adjunct therapies, such as hyperthermia or cytostatic agents, on early wound healing?

- How does irradiation of the anastomosis, immediately after construction, affect early repair?

- What is the long-term outcome after intra-operative irradiation of intestinal anastomoses?

Chapters 2-7 describe the experiments performed in order to answer these questions.

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Chapter 2

HIGH-DOSE PREOPERATIVE IRRADIATION WITHOUT DETRIMENTAL EFFECT ON EARLY REPAIR OF ANASTOMOSES IN THE COLON OF THE RAT

J.Biert[*], J.Hoogenhout[#], Th.Wobbes[*], Th.Hendriks[*]

From the Departments of [*]Surgery and [#]Radiotherapy, University Hospital Nijmegen, the Netherlands

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ABSTRACT

Purpose preoperative radiotherapy as an adjunct to surgery for rectal carcinoma is generally thought to impair the healing of colorectal anastomoses To delineate the presumed hazards of preoperative irradiation we investigated this effect in a new model where, contrary to experiments reported so far, anastomoses were constructed using normal tissue for the proximal limb and irradiated tissue for the distal limb

Methods and materials a group of 120 male Wistar rats, randomly divided into 12 groups of 10 each, was used In 60 animals, a colonic segment of 2 2 cm was irradiated with a single dose of 25 Gy administered 28 or 5 days (n=20 each), or 3 or 1 day(s) (n=10 each) before colonic resection For each experimental group, a control group was included which was sham-irradiated on the same preoperative day The animals were sacrificed on the third (n=10 in all groups) or on the seventh postoperative day (n=10 in groups of 20 rats), and healing of the anastomosis was evaluated by measurement of bursting pressure, breaking strength and hydroxyproline concentration and content

Results comparison between each experimental group and its control group showed that preoperative irradiation did not reduce strength of the anastomoses Also, hydroxyproline concentration and content of the anastomoses were unchanged

Conclusions these data indicate that construction of a colonic anastomosis consisting of one irradiated bowel end in rats is not by definition detrimental to the development of early wound strength

INTRODUCTION

Preoperative radiotherapy is frequently used as an adjuvant therapy to surgery for rectal cancer. Since local recurrence of rectal cancer is a disaster which is extremely difficult to treat, many clinical studies have been performed, both retrospectively and prospectively, to determine the efficacy of this adjuvant therapy for the prevention of local recurrent tumour growth (1-3). There is no consensus about its application since there appears a price to be paid in the form of unwanted side-effects like early or late irradiation injury of the normal intestine and surrounding tissues. In addition, it may be hazardous to perform intestinal resections with construction of a low anterior anastomosis. According to earlier studies there exists a significant chance of failure of anastomoses by leaking, or other problems like stenosis, bleeding or fistulae formation (4-6). In a large study by Schrock preoperative irradiation was identified as an important factor contributing to leakage from an anastomosis in the colon (7), a finding which could not be confirmed by others (8). Experimental evidence suggests that healing of

the anastomoses, as assessed by the development of strength, is indeed impaired by preoperative irradiation (9-12). However, results from a recent study suggest that this is not always the case (13). Apparently, the precise definition of the experimental details is important in determining the outcome of studies designed to investigate (negative) radiation effects on the repair of anastomoses. These details include the time interval between irradiation and surgery, the time of assessment of the anastomosis, and irradiation and surgical procedures.
The present experiment was designed to investigate whether a single high-dose irradiation shortly before operation affects early repair of an anastomosis, as measured by wound strength and collagen content.
Contrary to the experimental work available so far, we constructed the anastomoses using only one irradiated bowel end. This certainly reflects the clinical situation better than an anastomosis with both limbs consisting of previously irradiated tissue. Since the strength of an anastomosis is relatively low (14), and chances for anastomotic dehiscence are relatively high during the first days after operation, we paid particular attention to healing at the third postoperative day.

METHODS AND MATERIALS

Animals
120 Young adult male outbred Wistar/Cpb:WU rats were used. They received water and standard laboratory food (diet AM II, Hope Farms, Woerden, The Netherlands) ad libitum. The rats were randomly divided into 4 groups of 10 and 4 groups of 20 each (Table II-1), and all animals underwent colonic resection and construction of anastomosis; they were killed 3 days (10 in each group) or 7 days (10 in each group of 20 rats) after surgery. In rats from groups I, III, V, and VII, part of the colon was irradiated 1, 3, 5 or 28 days prior to operation, respectively. Animals from groups II, IV, VI and VIII served as controls and were sham-irradiated at the same days (Table II-1).
This study was approved by the Animal Ethics Review Committee of the Faculty of Medical Sciences, University of Nijmegen.

Irradiation and dosimetry

The procedure was based on techniques developed for a prior experiment (15). In order to ascertain that the same tissue area was irradiated in each rat, and to mark this area for subsequent surgery, animals were anaesthetized with intra-peritoneal sodium pentobarbital, and a median laparotomy was performed. The colonic segment to be irradiated, from 1 cm to 3.2 cm proximal to the recto-peritoneal fold (Figure II-1), was marked by a serosal stitch at its proximal border. The irradiated area measured 2.2 x 0.5 cm^2. The adjacent bowel and other organs were covered with a lead cone and the rest of the body was also shielded with lead (thickness 2.5 mm). Radiation dosimetry was performed by means of thermoluminescent dosimeters and film densitometry in separate animals. Irradiation was performed with a 250 kVp X-ray unit with a 1 mm Cu filter (target-colon distance 25 cm). The dose rate was 1.14 Gy/min. Thus, all rats in groups I, III, V and VII received 25 Gy. After irradiation was completed, the abdomen was closed with a running suture for the fascia and staples for the skin. Animals in the control groups (II, IV, VI and VIII) underwent the same procedure without irradiation (sham irradiated animals).

Table II-1. *Treatment schedule*

	irradiation	sham	sacrifice (n)	
			day 3	day 7
group I	day -1		9	-
group II		day -1	9	-
group III	day -3		8	-
group IV		day -3	10	-
group V	day -5		9	10
group VI		day -5	9	10
group VII	day -28		10	9
group VIII		day -28	9	10

Rats in all groups underwent colonic resection and anastomotic construction at day 0

Operative procedure

Animals were anaesthetized with intra-peritoneal sodium pentobarbital. A laparotomy was performed through the old scar and a 1.6 cm colonic segment was resected. This segment was identified by locating the marking stitch left during the irradiation procedure and measuring 0.5 cm in proximal and 1.1 cm in distal direction. The distal 0.5 cm of this segment was used as the control segment and kept for further hydroxyproline analysis (Figure II-1). Continuity was restored by an inverting one-layer end-to-end anastomosis with 8 interrupted mono-filament sutures (Ethilon 8-0, Ethicon®, Norderstedt, Germany) using microsurgical techniques.

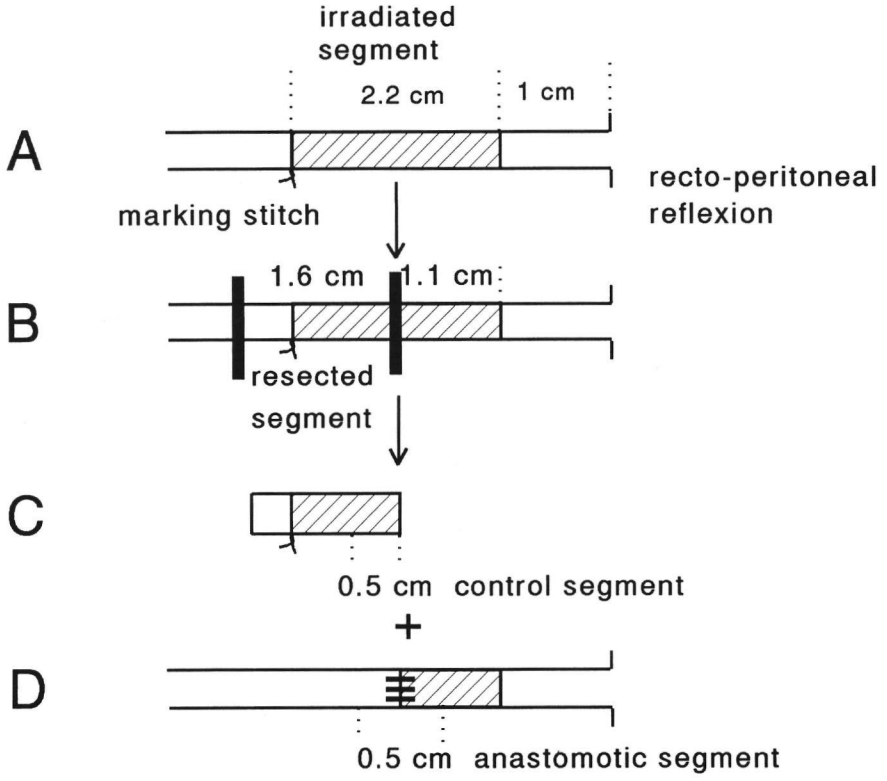

Figure II-1. Schematic representation of the experimental procedure. **A**. Irradiation of colonic segment. **B**. Resection of control segment and construction of anastomosis with only the distal limb consisting of irradiated tissue. **C**. Part of the resected colon is taken as the control segment and used for hydroxyproline analysis. **D**. At sacrifice, the anastomotic segment, containing the suture line in the middle, is also taken for hydroxyproline analysis.

Thus, the proximal limb to be used for the anastomosis consisted of normal tissue, while the first 1.1 cm of the distal limb had been irradiated (in groups I, III, V and VII). Fascia and skin were closed with a running suture and staples, respectively.

Analytical procedures

The condition of the animals was monitored and weight was measured daily or weekly. Three or seven days after operation the rats were killed by cardiac puncture. The abdomen was inspected for rectal stenosis or other abnormalities. The anastomoses were resected en bloc. Healing was assessed by measurement of strength of the anastomsis and hydroxy-proline content (14). In order to determine wound strength, the anastomotic segment was washed in saline and connected to an infusion pump on one side and to a manometer on the other side. The bursting pressure was measured by raising the intra-luminal pressure by infusion of a methylene blue/saline solution at a rate of 2 ml/min. The procedure was performed in water for better visualisation of the bursting site. The bursting pressure was defined as the maximum intra-luminal pressure the segment resisted, expressed in mm Hg. The bursting site was noted. The breaking strength of the segment, as a measure of the resistance to longitudinal forces, was measured immediately after measurement of the bursting pressure. The segment was placed in a tensiometer that measured the force, which occurred by pulling at one end. This pulling force was gradually increased with a constant speed. The peak force (in gram) necessary to induce disruption of the segment was taken as the breaking strength. The breaking site was noted. Thereafter, adhesions and fat tissue were removed from the segment and a 5 mm sample containing the suture line was collected (Figure II-1) and stored in liquid nitrogen for a hydroxyproline assay.

Samples of anastomoses, and control segments removed at operation, were lyophilized, weighed, pulverized, and stored at -80°C. Subsequently, the hydroxyproline content, as a measure for collagen, was determined as described previously (16), essentially according to the method of Prockop and Udenfriend (17).

The primary variables in the statistical analysis are the bursting pressure, breaking strength and hydroxyproline concentration and content. To handle the problem of multiple comparisons and control the type I error the

Bonferroni procedure has been applied for each of the primary variables. For each primary variable 6 comparisons (two-tailed Mann-Whitney U tests between experimental groups and their controls) were carried out. In order to obtain an overall significance level of 0.05 for each variable a 0.05/6 = 0.008 level was used to judge the P-values of the tests.

RESULTS

General observations

Eight rats died because of anaesthesiological complications (depression of ventilation) during or immediately after (sham-)irradiation (n=4) or operation (n=4). As a consequence, 9 rats remained in groups I and II, 8 in group III, 10 in group IV, and 19 in groups V - VIII each. These rats tolerated the subsequent (sham-)irradiation and surgical procedures well. Moderate diarrhoea was seen in irradiated animals. Five and twenty-eight days after irradiation, during the operation of animals of groups V and VII, a thickening of the bowel wall was observed in the irradiated area. This was reflected in the dry weight of the control segments, which was 15.0 ± 2.2 (SD) mg in group V and 11.3 ± 3.0 mg in group VI; 4 weeks after irradiation this effect was even more pronounced: the dry weight was 13.2 ± 2 mg in group VII and 6.5 ± 0.7 mg in group VIII. One of the animals in group VII (3 days group) suffered from functional rectal stenosis, with a proximal colonic diameter of 1 cm. None of the animals showed signs of peritonitis. Although all animals had some peri-anastomotic adhaesions, they appeared to be more abundantly present in irradiated animals.

Both irradiation and surgery led to a transient loss in body weight (typical example: Figure II-2). After both procedures animals lost approximately 10-20 g of weight. However, the normal course of weight gain was resumed shortly after. No significant differences were found between irradiated and control groups.

Strength of anastomoses

Technical problems precluded the measurements of the strength of the anastomosis in one rat from group I. The individual bursting pressures measured in all other animals are represented in Figure II-3. On the average,

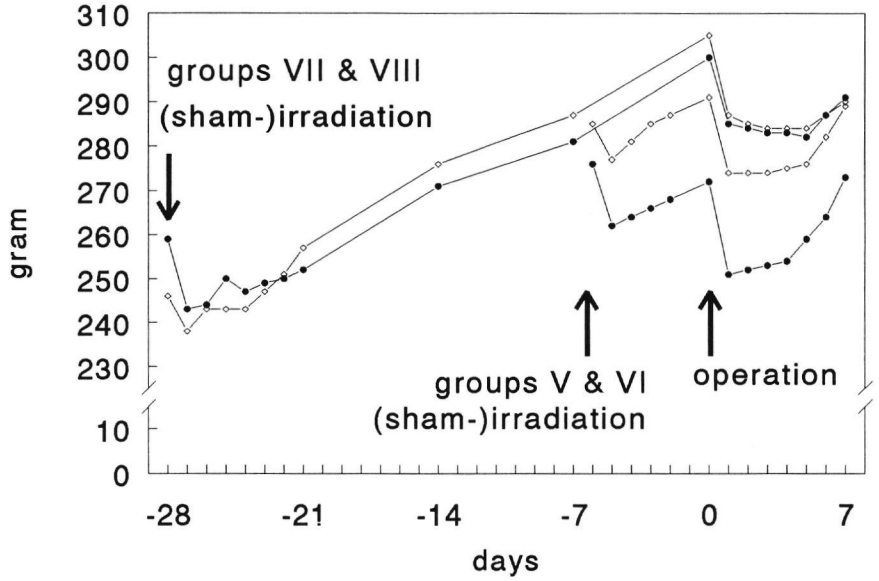

Figure II-2. Change in body weight during the experiment. Average values for groups V and VII (irradiated: closed circles) and group VI and VIII (sham-irradiated: open circles) are shown.

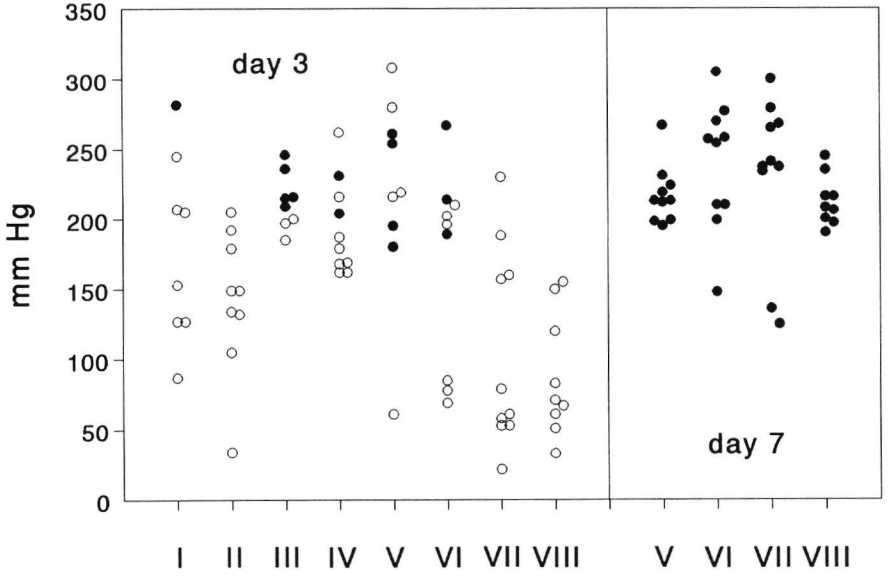

Figure II-3. Anastomotic bursting pressure. Each point represents a measurement in a single animal. Groups I,III,V,VII: irradiation 1, 3, 5 or 28 days before operation, respectively. Groups II,IV,VI,VIII: sham-irradiation 1, 3, 5 or 28 days before operation, respectively. Open circles: bursting site within suture line; closed circles: bursting site outside suture line.

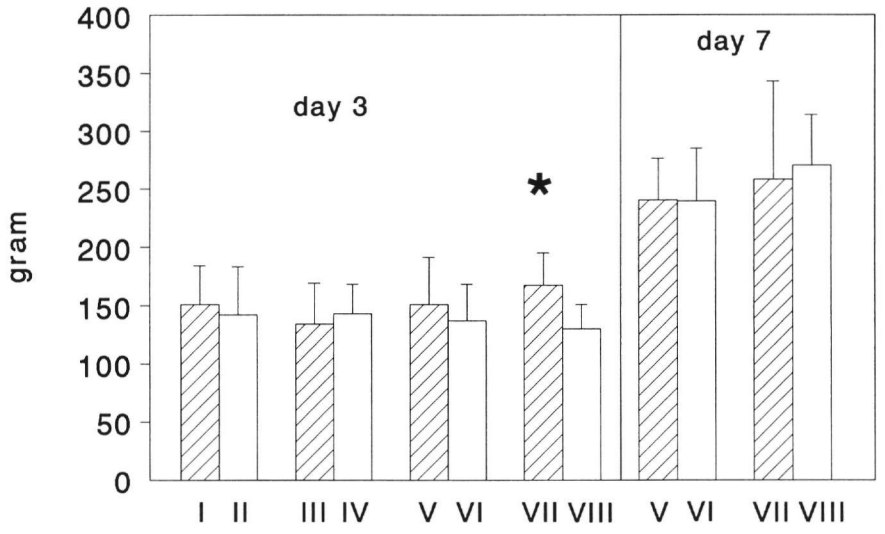

Figure II-4. Anastomotic breaking strength. Bars represent average values + SD. Groups were irradiated 1 (I,II), 3 (III,IV), 5 (V,VI) or 28 (VII, VIII) days before operation. Striped bars: irradiation. Open bars: sham-irradiation.
*: $P<0.008$ (Mann-Whitney U test) difference vs control group.

bursting pressures were somewhat higher in the irradiated groups than in their respective control group. Three days after operation the mean bursting pressure in group I, irradiated 1 day before operation, was 179 ± 66 (SD) mg Hg, compared to 142 ± 51 mm Hg in control group II. In the groups irradiated 3 (III) and 5 (V) and 28 (VII) days prior to surgery, the mean values were 213 ± 20, 219 ± 72 and 106 ± 71 mm Hg, respectively, while the values in the corresponding control groups (IV, VI and VIII) were 194 ± 34, 168 ± 71 and 88 ± 44 mm Hg, respectively. These differences between irradiated and their control groups were not statistically significant.
In the majority of cases, the bursting site was within the suture line. The number of cases where the bursting site was outside the area of the anastomosis was slightly higher in the irradiated groups than in the control groups (10 vs. 5). This indicates that the anastomosis had grown more resistant to intra-luminal pressure than the adjacent bowel wall in these groups.
In the groups killed seven days after operation, average bursting pressures were 217 ± 21 (V), 222 ± 22 (VI), 244 ± 46 (VII) and 203 ± 32 (VIII) mm Hg respectively (differences between control and irradiated groups not

significant). These values reflected bursting resistence of nearby tissues, since the bursting site was only once (group VII) inside the anastomosis, and are still somewhat below those measured for non-irradiated, non-surgically treated, normal large bowel (280 ± 46 mm Hg, n=10, unpublished results).
Three days postoperatively, measurement of the breaking strength invariably yielded a breaking site within the anastomosis. Figure II-4 shows that the average breaking strength in irradiated and control groups were similar in groups I-VI. In group VII the breaking strength was significantly increased as compared to group VIII: 167 ± 28 versus 130 ± 21 gram (P=0.0021).
After seven days rupture occurred ouside the anastomosis in 7 out of 10 (V), 3/10 (VI), 5/10 (VII) or 3/10 (VIII) cases. Average values in control and irradiated groups were not significantly different.

Hydroxyproline concentration and content
Average values for the hydroxyproline concentration and content in the control segments are shown in Figure II-5. While no differences were found between irradiated and normal colonic tissue 1 or 3 days after irradiation, it appeared that 5 and 28 days after irradiation the hydroxyproline content had increased significantly (V vs. VI: P=0.003; VII vs. VIII: P<0.0001; Mann-Whitney U test) in animals killed 3 days later. A comparible difference was found in animals killed 7 days later in group VII vs. VIII (P<0.0001).
Figure II-6 shows the hydroxyproline concentration and content of the anastomotic segments. In most irradiated groups, the average hydroxyproline concentration was slightly elevated in comparison to the control groups. In group VII, irradiated 4 weeks before the operation, hydroxyproline concentration and content were even significantly increased 7 days postoperatively (P<0.0001 and P=0.0004 respectively, Mann-Whitney U test).

DISCUSSION

The present data show that preoperative irradiation, delivered in a single dose between 1 and 28 days before colonic resection, does not impair the development of strength of the anastomosis shortly after resection.

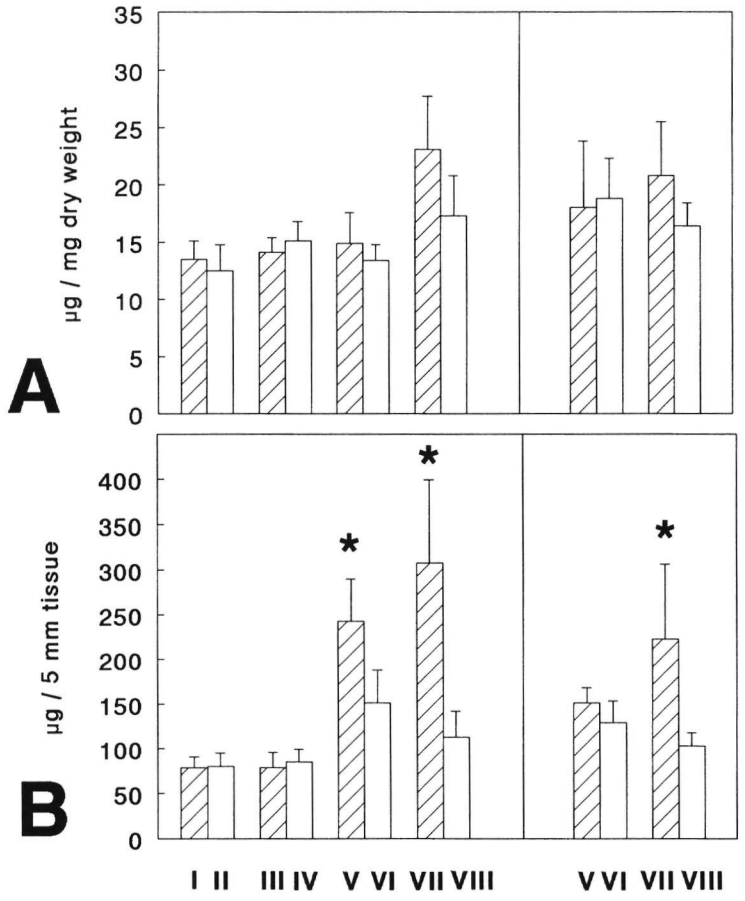

Figure II-5A,B. Hydroxyproline concentration (**A**) and hydroxyproline content (**B**) of control segments, removed at operation. Data represent average values + SD. Groups were irradiated 1 (I,II), 3 (III,IV), 5 (V,VI) or 28 (VII,VIII) days before operation. Striped bars: irradiation. Open bars: sham-irradiation.
*: $P<0.008$ (Mann-Whitney U test) difference vs. control group.

Application of radiotherapy in treatment of rectal cancer has been advocated to prevent local recurrence and to downstage the tumour (2,3).
Since the use of radiotherapy is limited by the tolerance dose of intestine, ureter, nervous system and other organs which may be irradiated sideways, it is not possible to gain total tumour necrosis by radiotherapy alone. Irradiation therefore must be seen as an adjunct to surgery. Peroperative tumour spill is said to decrease, while local tumour control increases (2,3). Although radiotherapy has definite advantages in these patients, there are

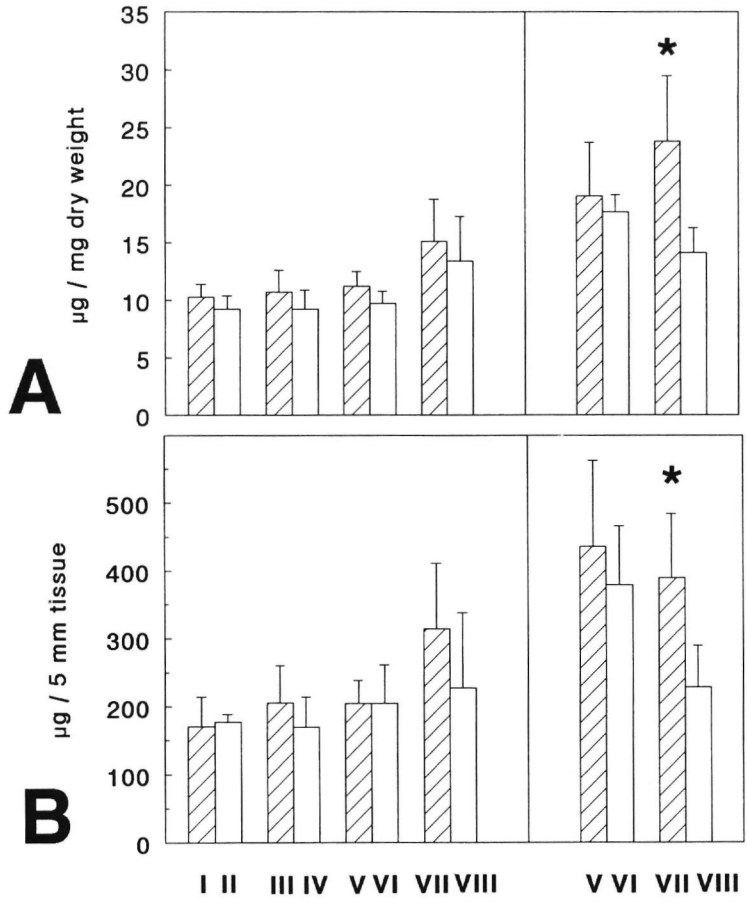

Figure II-6A,B. Hydroxyproline concentration (**A**) and hydroxyproline content (**B**) of anastomotic segments. Data represent average values + SD. Groups were irradiated 1 (I,II), 3 (III,IV), 5 (V,VI) or 28 (VII,VIII) days before operation. Striped bars: irradiation. Open bars: sham-irradiation.
*: $P<0.008$ (Mann-Whitney U test) difference vs control group.

believed to be serious side effects which may be of importance for the near and distant future of the patient. At first there are the early side effects which may present in the first few days or weeks after surgery, especially after rectal excision and construction of a low anastomosis: impaired healing of the anastomosis and early rectal stenosis.
After a longer period of time, even after many years, late side effects may become manifest like ureteral stenosis, neuropathy, and irradiation damage to the small and large bowel.

Problems are bleeding, ulceration, fistulisation, stenosis and fibrosis (6,18-24). The rat model is generally considered to be a good model for studying these irradiation effects (21).
The results from our experiment indicate the possibility of undisturbed healing of anastomoses after a single high irradiation dose. Strength of the anastomoses soon after resection, as measured by either bursting pressure or breaking strength, might even be slightly higher after preoperative irradiation.
This is significantly so for the breaking strength at 3 days-old anastomoses constructed 28 days after irradiation and could be caused by the notably increased collagen content observed in the area of the anastomosis. Apparently, a fibrotic response to irradiation of the bowel wall has no negative effect on early development of strength of the anastomoses.
In the current study we employed a control group for each experimental group. Although the various control groups (II, IV, VI and VIII, respectively) generally showed similar breaking strength of the anastomoses and hydroxyproline concentration, differences between these groups also occurred, particularly in bursting pressure. Three days after operation, the bursting pressure in group VIII, which was sham-irradiated 28 days prior to surgery, was lower than that in the other control groups. Apparently, performance of such a procedure, including laparatomy under full anaesthesia, four weeks before construction of anastomoses somehow affects wound repair. It is noteworthy that the average bursting pressure in irradiated group VII is also lower than that of the other irradiated groups. This result underlines the necessity for including the proper controls in this type of experiment.
The findings in our experiment seem to be in contrast with most of the results from reported studies on healing of anastomoses after preoperative irradiation in the rat. Various authors, using irradiation doses between 2 and 20 Gy applied 1-15 days before construction of an anastomosis, have reported profound negative effects on bursting pressure of anastomoses (9-11) or breaking strength (12), measured 5-7 days after operation. A major concern about the studies where bursting pressure is measured as a parameter for strength of anastomoses (9-11) is that no mention is made of the bursting site. The bursting pressure is a measure for strength of an anastomosis only if rupture occurs within the suture line. In general, the site

of rupture shifts outside the area of the anastomosis from 4 days after operation onwards (14). Studies which include measurements of the bursting pressure after this time point should include data on the bursting site in order to allow a correct interpretation of the numerical data. The first few days of healing are crucial to repair of anastomoses: wound strength is low and chances for dehiscence relatively high. From 3 days after the operation onwards, strength of the anastomosis rises significantly. For this reason, we focussed our attention at the third postoperative day. In the groups irradiated 28 and 5 days prior to surgery we extended the measurements to 7 days and found a development of strength of the anastomoses similar to that in the respective control groups.

It is very interesting that Weiber *et al.* reported recently that administration of 2 subsequent doses of 10 Gy, 4 and 8 days before operation, does not affect the breaking strength of the anastomoses (13). Their conclusion, that preoperative irradiation not necessarily compromises healing of the anastomoses, is supported by our results. In addition, Jahnson *et al.* (25) found that chronic radiation damage of the small bowel, induced by irradiation 20 weeks prior to surgery, did not reduce strength of anastomoses. It increased the frequency of complications due to changes in the tissues around the anastomoses, like adhesion formation and the incidence of purulent intra-abdominal abscesses. In our model, we found no signs of peritonitis at all, although some adhesion formation was always observed. Undoubtedly, the conflicting results in the various studies are also caused by major differences in experimental protocol, particularly with respect to irradiation dose and method, and the use of irradiated tissue for construction of anastomoses.

We chose a dose of 25 Gy in our study in order to investigate the effects of radiation in a dose range where one would expect healing of the anastomosis to be negatively affected. When looking at the human situation, the tolerance dose (TD 5% severe complications in 5 years) for acute effects of human intestine is approximately 50 Gy in daily fractions of 2 Gy. According to the linear quadratic concept and assessing an α/β ratio of 10 Gy for acute effects, the extrapolated tolerance dose of 60 Gy is approximately the biological equivalent for acute effects with a single dose of 20 Gy (26-28). While a single dose of 25 Gy is above the accepted tolerance dose for human intestine, it is at the upper limit for rat intestine (19-21).

The size of the irradiated volume plays an important role in deciding the amount of damage (28). Thus, accurate preoperative localisation of the colon resection site is of the utmost importance to ensure that one limb of the future site of the anastomosis is irradiated, and that the remainder of the irradiated field is limited. After a pilot study optimizing irradiation procedures we changed from a closed to an open irradiation technique in order to be sure that only one limb of the anastomosis was irradiated, which in our view resembles mostly the human situation after a low anterior resection. This procedure is essentially different from that followed by others (5,9-13) where both limbs used for construction of the anastomosis consisted of irradiated tissue. Experimental studies with one versus both limbs of the anastomosis being irradiated intra-operatively have been performed both for large bowel, in our own laboratory (29), and for small bowel (30). In both cases, negative effects on strength early after the resection and other healing parameters were more extensive if both limbs of the anastomosis were irradiated.

For practical reasons, the number of animals in both the control and experimental groups was set at 10. This limited sample size was deemed sufficient to allow detection of major, physiologically and clinically significant, differences between groups. However, not finding a statistically significant difference for a primary variable does not preclude entirely the presence of a minor, and in our opinion physiologically less relevant, difference between experimental and control groups. In order to control for type I errors, we have applied the Bonferroni procedure, thereby increasing the likelyhood of such a type II error. Both types of error can only be kept low by using prohibitively large numbers of animals.

In conclusion, although our experiment showed no direct effect of preoperative radiation therapy on strength of the anastomosis soon after resection, this does not necessarily mean that there will be no effect at all. Since radiation colitis can be present after a dose of 25 Gy, late effects in the area of the anastomosis might possibly be expected (28). A well known phenomenon is the forming of pseudo-obstruction with rectal stenosis (20,22). It remains to be determinated if such late or long-term side-effects of preoperative irradiation indeed occur.

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Chapter 3

COMBINED PREOPERATIVE IRRADIATION AND DIRECT POSTOPERATIVE 5-FLUOROURACIL WITHOUT NEGATIVE EFFECTS ON EARLY ANASTOMOTIC HEALING IN THE RAT COLON

J.Biert[*], Th.Wobbes[*], J.Hoogenhout[#], B.de Man[*], Th.Hendriks[*]

From the Departments of [*]Surgery and [#]Radiotherapy, University Hospital Nijmegen, the Netherlands

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ABSTRACT

Background and Purpose: Preoperative irradiation with direct postoperative chemotherapy could benefit patients undergoing surgery for colorectal cancer. This study was designed to examine, in an experimental model, if such treatment is feasible without detrimental effects on early anastomotic healing.
Material and Methods: a colonic segment was irradiated (25 Gy) in 3 groups (n=10 each) of male Wistar rats. After 5 days a colonic resection was performed with anastomotic construction; only the distal limb consisted of irradiated bowel. Postoperatively, animals received daily intraperitoneal 5-fluorouracil (5-FU, group I/CH: 17.5 mg/kg; group I/CL: 12.5 mg/kg) or saline (group I). Three additional groups were treated similarly, but with sham-irradiation: CH, CL and C, respectively. All rats were killed 7 days postoperatively. Parameters measured were: weight, serum albumin and protein, and anastomotic bursting pressure, breaking strength and hydroxyproline content.
Results: body weight was diminished significantly in rats receiving chemotherapy. Serum albumin and protein was significantly lower in irradiated groups. At sacrifice 40% of I/CH rats had functional rectal stenosis. The average bursting pressure (P=0.0005) and the average breaking strength (P=0.012) were only reduced significantly in the CH group. The anastomotic hydroxyproline content was significantly higher in the I/CH and I/CL groups vs. the control group.
Conclusion: high-dose direct postoperative 5-FU leads to reduced anastomotic strength. Although the combination of preoperative irradiation (25 Gy) and direct postoperative high-dose 5-FU does not reduce early anastomotic strength, some stenosis may occur. The combination of preoperative irradiation and low-dose 5-FU has no such effect.

INTRODUCTION

Local recurrence of colorectal cancer and metastatic disease after surgery are common entities; they are due to microscopic disease left after resection, peri-operatively spilled tumor cells or unrecognised metastatic disease at the time of operation. Over the last decades numerous studies have been performed, with limited succes to determine the role of adjuvant therapy in improving the results of surgery. Radiotherapy and chemotherapy are commonly used tools for adjuvant treatment, and there exists increasing interest in combining both modalities (1).
The best setting for radiation treatment seems to be a high-dose preoperative application (2). The potential advantages for such a schedule are many. Firstly, irradiation is delivered to a well vascularized tumor which is well oxygenized, providing a better therapeutic effect. Secondly, postoperative

intra-abdominal adhesions, resulting in radiation side effects to the fixed small bowel, have not been formed. Thirdly, the operative wound after surgery would require a much larger irradiation field, giving rise to volume effects. Finally, implantation of tumor cells spilled during surgery will be less because they are less viable, and because the tumor is reduced in size. Postoperative chemotherapy is believed to be effective in the attack on viable cancer cells, resulting in decreased local recurrence, increased disease-free interval and increased survival both clinically (3,4) and experimentally (5). 5-Fluorouracil (5-FU) is still considered to be the most effective single agent in the use against colonic cancer cells (6,7).
Separately, both types of adjuvant therapy have been thought to endanger the healing of bowel anastomoses (8-14). Early disturbances of anastomotic healing may lead to leakage, peritonitis and death, thus forming a threat which might limit their use. However, the precise magnitude of these side effects is still under investigation. Irradiation has long been considered to be detrimental for colon anastomotic repair (9-12), but recent data (2,15-17), also from our own laboratory (1) indicate that it can be applied without negative effects on early anastomotic strength or anastomotic integrity. With respect to chemotherapy, it has been shown that in the rat early bowel anastomotic repair is endangered by a high dose of 5-FU, given daily for one week from the day of the operation onwards (8,14). If treatment is limited to the first three days, a high dose can be considered safe (19,20), unless 5-FU is used together with other agents (13). Lower doses of 5-FU are supposedly less dangerous for colon anastomotic repair.
However, the possibilities for combining two treatment modalities may be limited due to amplification of side effects (21). So far, the possible interactions of preoperative irradiation and direct postoperative chemotherapy with respect to their effects on early anastomotic healing have not been investigated. Therefore, we examined the effect of combined preoperative irradiation with a dose of 25 Gy and direct postoperative 5-FU, administered intraperitoneally in a high or low dose, on anastomotic healing in the rat colon. The effect of combined treatment was compared to that of either single treatment modality.

MATERIALS AND METHODS

Animals

Sixty young adult male outbred Wistar/Cpb:WU rats, body weight 246 ± 16 gram, were used. They received water and standard laboratory food (diet AM II, Hope Farms, Woerden, The Netherlands) ad libitum. The rats were randomly divided into 6 groups of 10 animals each. A part of the colon was (sham-)irradiated preoperatively with 25 Gray X-rays; after 5 days a colonic resection was performed with anastomotic reconstruction. Postoperatively rats received 5-fluorouracil (5-FU) intraperitoneally daily, until sacrifice at day 7 after surgery. Rats in group C served as sham-treated controls (sham-irradiation plus postoperative saline). Groups CH and CL were sham-irradiated and received postoperative high- and low-dose chemotherapy (dose: see below), respectively. Group I was irradiated and received postoperative saline, while groups I/CH and I/CL were treated by irradiation and postoperative high- and low-dose chemotherapy, respectively.
This study was approved by the Animal Ethics Review Committee of the Faculty of Medical Sciences, University of Nijmegen.

Irradiation and dosimetry

Before irradiation the animals were anaesthetized with intraperitoneal sodium pentobarbital. The irradiation procedure was based on techniques developed in a prior experiment (18). In order to ascertain that the same tissue area was irradiated in each rat, and to mark this area for subsequent surgery, a laparotomy was performed. The colonic segment to be irradiated, 1 - 3.2 cm proximal from the rectoperitoneal fold was marked by a serosal stitch at its proximal border. The irradiated area measured 2.2 x 0.5 cm^2 (Figure III-1). The adjacent bowel and other organs were covered with a lead cone and the rest of the body was also shielded with lead (thickness 2.5 mm). Radiation dosimetry was performed by means of thermoluminescent dosimeters and film densitometry in separate animals. Irradiation was performed with a 250 kV X-ray unit with a 1 mm Cu filter (target-colon distance 25 cm). The dose rate was 1.29 Gy/min. Thus, all rats in groups I/CH, I/CL and I received a dose of 25 Gy.

Operative procedure and chemotherapy

After 5 days animals were anaesthetized again with intraperitoneal sodium pentobarbital. The median laparotomy wound was opened and a 1.6 cm colonic segment was resected. This segment was identified by the marking stitch left during the initial laparotomy and measured 0.5 cm in proximal and 1.1 cm in distal direction (Figure III-1). Thus, the proximal limb to be used for the anastomosis consisted of non-irradiated tissue, while the first 1.1 cm of the distal limb had been irradiated (groups I/CH, I/CL and I). Continuity was restored by an inverting one-layer end-to-end anastomosis with 8 interrupted monofilament sutures (Ethilon 8-0, Ethicon®, Norderstedt, Germany) using microsurgical techniques. Fascia and skin were closed with a catgut running suture and staples, respectively.

Chemotherapy was started immediately after operation, and the intraperitoneal way of administration was chosen on the basis of earlier work of our group (14,20): 5-FU, administered intraperitoneally from the day of operation onwards in a daily dose of 20 mg/kg body weight - which is the maximum tolerated dose - strongly reduced strength and accumulation of collagen in 7 days old intestinal anastomoses in the rat (14). Based on these findings, groups I/CH and CH (high-dose groups) now received 17.5 mg 5-FU/kg body weight intraperitonally in saline in a single dose every day until sacrifice. Groups I/CL and CL (low-dose groups) received 12.5 mg FU/kg body weight daily, while in groups I and C the same volume of saline was given every day as in group I/CH (sham medication treatment).

Analytical procedures

The condition of the animals was monitored and weight was measured daily. Seven days after the operation the rats were killed by cardiac puncture. A blood sample was taken for albumin and protein measurement to monitor general nutritional condition. The abdomen was inspected for adhaesions, rectal stenosis, abcesses or other abnormalities. The anastomoses were resected en bloc. Healing was assessed by measurement of anastomotic strength and hydroxyproline content (22). In order to determine anastomotic strength, the anastomotic segment was washed in saline and connected to an infusion pump on one side while the other side was clamped. A manometer was connected by a side line. The bursting pressure was measured by raising the intraluminal pressure by infusion of a methylene blue/saline solution at

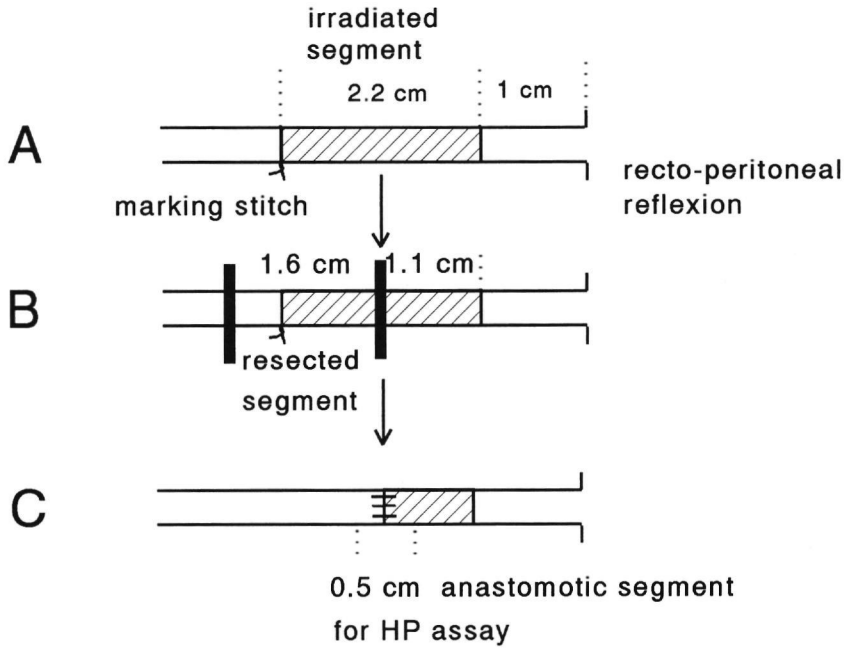

Figure III-1. Schematic representation of experimental procedure. **A**. First laparotomy plus irradiation of colonic segment. **B**. Second laparotomy plus resection of a 1.6 cm segment, and construction of anastomosis with only the distal limb consisting of irradiated tissue. **C**. At sacrifice: sample used for hydroxyproline analysis.

a rate of 2 ml/min. The procedure was performed in water for better visualisation of the bursting site. The bursting pressure was defined as the maximal intra-luminal pressure the segment resisted, expressed in mm Hg. The bursting site was noted. The breaking strength of the segment, as a measure of the resistance to longitudinal forces, was measured immediately after determination of the bursting pressure (13). The segment was placed in a tensiometer that provided a constantly increasing distraction. The peak force (in g) necessary to induce total disruption of the segment was taken as the breaking strength. The breaking site was noted. After this, adhesions and fat tissue were removed from the segment and a 0.5 cm sample containing both sides of the suture line was collected (Figure III-1) and stored in liquid nitrogen for hydroxyproline assay.

Anastomotic samples, and control segments removed at operation, were weighed, lyophilized, and pulverized. The hydroxyproline content, as a measure for collagen, was measured by HPLC after hydrolysis with 6N HCl

and derivatisation with dabsylchloride.
Differences between the control group and the five experimental groups were tested for significance using a two-tailed Mann-Whitney U test. To correct for the fact that multiple comparisons were made, pairwise comparisons were done using a level of significance of $\alpha'=2\alpha/k$ where k is the total number of pairwise comparisons. Thus, differences between groups were considered significant ($\alpha=0.05$) at $p<\alpha'$, where $\alpha'=0.02$.

RESULTS

General observations
All animals tolerated chemotherapeutic treatment and/or radiotherapy well. Moderate to severe diarrhea was apparent in all but the C groups. One animal in the I/CH group died on the sixt postoperative day - obduction revealed no abnormalities. One rat in group CL was moribund at the time of sacrifice. Functional rectal stenosis was defined as a 2 times or more enlarged proximal anastomotic limb diameter with faecal impaction, although passage of stool was still possible; this condition was seen in 4 rats in group I/CH and in 1 rat in group I/CL.
Anastomotic abcesses were seen once in groups I/CH, I/CL and CH each.

Body weight
After the first anaesthesia, all rats lost some weight: recovery seemed slower in the irradiated groups (Figure III-2). The second anaesthesia again resulted in weight loss, which was similar in all groups on the first postoperative day. Thereafter, all rats under 5-FU medication lost progressively more weight, the high dose groups most. Rats treated with sham medication gained weight again. In comparison with the control group differences were significant in the I/CL group from day 7 onwards, in groups CH and I/CH from day 8 onwards and in the CL group from day 11 onwards. The I group showed no significant differences with the control group.

Serum albumin and protein
Serum albumin and protein measurements are depicted in Table III-1. At sacrifice there were significant differences between all irradiated groups and

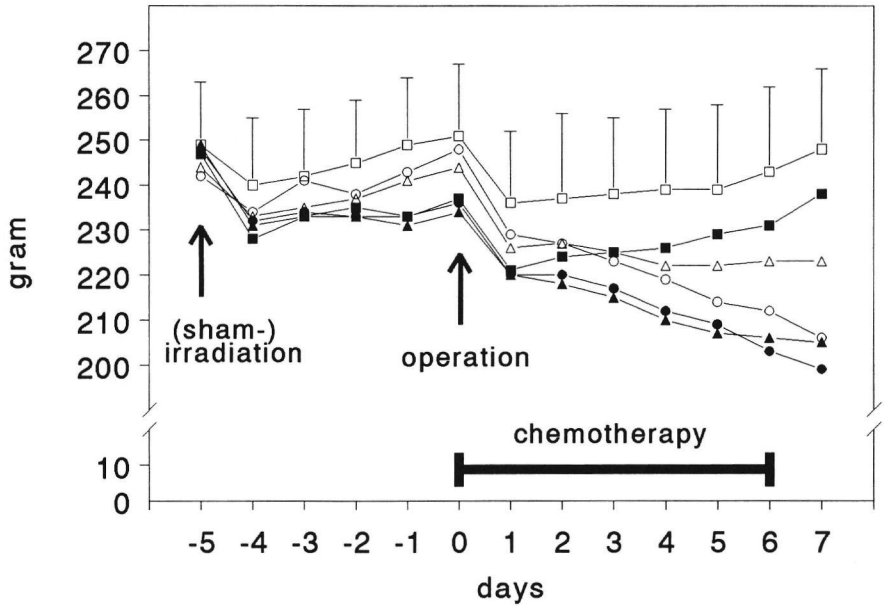

Figure III-2. Body weight as a function of time after (sham-)irradiation. Open squares: C group (+ SD); open triangles: CL group; open circels: CH group; closed squares: I group; closed triangles: I/CL group; closed circels: I/CH group.

the control group (except for albumin in the I group); this difference appeared progressively more pronounced in groups I/CL and I/CH. The CH group also showed lower values for serum protein.

Anastomotic healing

Individual bursting pressure measurements and bursting sites are depicted in Figure III-3. Seven days after operation the bursting site was almost invariably outside the suture line in both the C and I groups. Treatment with 5-FU led to weakening of the anastomotic bursting pressure, as indicated by a shift in bursting site to the anastomotic area and reduced mean values in CL and CH (P=0.0005; Mann-Whitney U test) groups. Groups which had received the radiation dose all showed higher average bursting pressures than their sham-irradiated counterparts.

Breaking strength measurements are depicted in Table III-2. Mean values were higher in the I group as compared to the control group, and lower in all other groups. Statistical significance was reached in the CH group only (P=0.012; Mann-Whitney U test). The site of the anastomotic rupture was always located inside the anastomosis, except for 1 rat in the I/CL group and 3 rats in the I group.

Table III-1. *Serum albumin and protein (mean ± SD) at sacrifice*

Group	Serum albumin *g/l*	Serum protein *g/l*
C	24 ± 2.8	55 ± 4.2
CL	25 ± 1.7	55 ± 3.8
CH	23 ± 1.4	50 ± 3.1*
I	22 ± 1.2*	51 ± 2.4*
I/CL	21 ± 1.0*	50 ± 2.7*
I/CH	20 ± 2.2*	47 ± 3.2*

*: P<0.02 (Mann-Whitney U test) difference vs control group

Hydroxyproline concentration and content of anastomotic samples are depicted in Table III-2. There were no significant differences in hydroxyproline concentration between experimental groups and the control group. The mean hydroxyproline content was increased in all but the CL

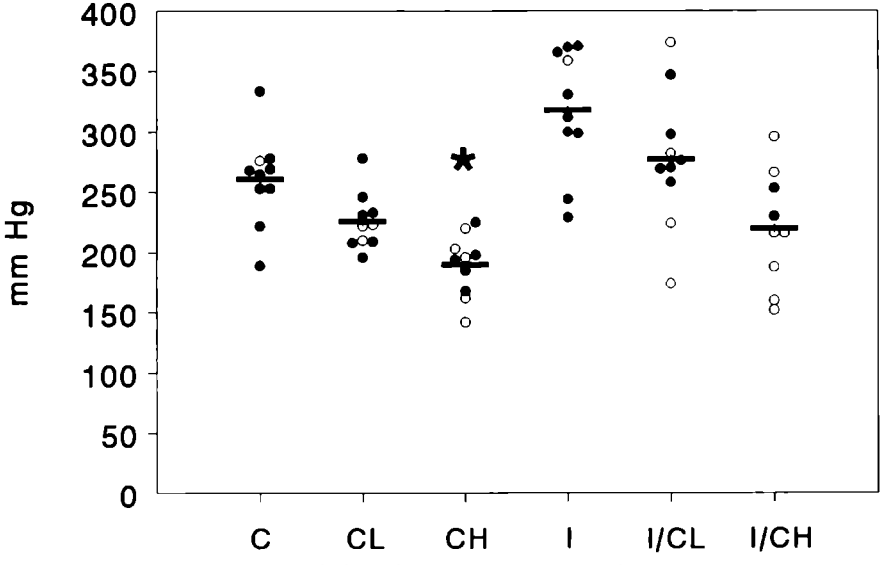

Figure III-3. Average anastomotic bursting pressure. Each point represents a measurement in one single animal. Open circles: bursting site within the anastomosis; closed circles: bursting site outside the anastomosis.
*: P=0.0005 (Mann-Whitney U test) difference vs. control group.

Table III-2. *Breaking strength, hydroxyproline concentration, and hydroxyproline content of anastomotic segments (average ± SD)*

group	breaking strength *gram*	hydroxyproline concentration *µg/5 mm tissue*	hydroxyproline content *µg/mg*
C	217 ± 62	16.4 ± 1.7	331 ± 51
CL	182 ± 54	15.2 ± 1.8	305 ± 75
CH	156 ± 41#	18.3 ± 3.5	403 ± 168
I	254 ± 41	18.3 ± 2.0	421 ± 96
I/CL	177 ± 65	18.8 ± 4.3	497 ± 120*
I/CH	175 ± 47	14.8 ± 2.9	498 ± 82*

#: P=0.012, *: P=0.0015 (Mann-Whitney U test) difference vs. control group

groups. The difference with the control group reached statistical significance in the I/CH group (P=0.0003) and the I/CL group (P=0.0015).

DISCUSSION

In this study it is shown that high dose preoperative irradiation combined with a low dose of intraperitoneal 5-FU, applied in the immediate post-operative period, does not influence early strength of experimental colonic anastomoses. Although there were no statistical differences concerning anastomotic strength, reservations must be made for the application of a combination of preoperative irradiation with high dose 5-FU. Animals in the I/CH group lost much weight (during the experiment almost 20 %). This went together with a significantly decreased serum albumin and protein ($p=0.0003$ and $p<0.0001$ respectively). The high incidence of functional rectal stenosis (4 out of 9), with another animal dying for unknown reasons before the end of the experiment, raises doubts about the safety of this combination. The increased anastomotic hydroxyproline (collagen) content in the I/CH and I/CL groups suggests a strong fibrotic reaction, even stronger than the reaction seen after irradiation alone. This fibrosis does not

lead to increased early strength as compared to the control group. However, the possibility cannot be excluded that it might be the start of future rectal stenosis.

Clinical and experimental trials concerning surgery for colorectal cancer are increasingly focussed on the application of adjuvant therapies (1). Some clinical studies have dealt with combined effects of radiotherapy and chemotherapy as an adjunct to surgery, although so far a combination of preoperative irradiation and direct postoperative chemotherapy has not been reported yet. Graf describes undisturbed anastomotic wound healing after a seven days course of 5-FU plus leucovorin intraperitonally (23).

In a prospective study by Chari, in a group of 43 patients, a combination of 5-fluorouracil, cisplatin and preoperative irradiation with a dose of 45 Gy in fractions was given in a 5-week period prior to resection; this resulted in increased survival, decreased local recurrence and increased disease-free interval (3). Only 5 patients underwent a low anterior resection with anastomotic construction. No anastomotic leakage occurred in these patients.

The beneficial effects of the combination of postoperative irradiation with prolonged postoperative chemotherapy on survival and tumor recurrence, especially in patients stage Dukes B or C rectal carcinoma, has also been proven in two prospective randomized trials (4,24). However, nowadays adjuvant radiation therapy is generally thought to be most effective in the preoperative setting (2,26,27).

Adjuvant intraperitoneal chemotherapy is most useful in the first days after surgery. Since hardly any adhaesions have been formed the anastomotic site and surrounding pelvic wall have not yet been sealed off, allowing a wide spread of the drug at the right location (28). Also, high doses of regional chemotherapy over prolonged (120h) time periods should translate into a high fraction of cell kill and a small likelihood of drug resistance (28). Furthermore, 5-FU administered intraperitoneally is preferably taken up by the visceral peritoneum, resulting in a high concentration in portal venous blood, in this way working possibly more powerful against hepatic implantation of metastatic cells than systemic application (5,28).

The combination of radiotherapy and chemotherapy is being used more and more often to improve results of adjunct therapy to surgery for cancer, as is done in colorectal cancer patients. Application can be sequentially,

alternated or simultaneously (29). When a sequential order is followed, and 5-FU is administered first, synchronisation and radiosensitation occur. If radiotherapy is given first, more cells become sensitive to 5-FU (29). In cancer cell lines the effect was more pronounced if irradiation preceded 5-FU (29). In mice the same experiment showed that chronology of both adjunct therapy modalities was not important, although simultaneous application gave slightly better results (30).

In the present experiment we used maximally 17.5 mg 5-FU/kg body weight, aiming at the highest dose which would leave colonic repair essentially unaffected (14). Still, this regimen (the CH group) significantly reduced anastomotic strength, although without concomitant decrease in wound hydroxyproline content. The lower dose of 12.5 mg 5-FU/kg body weight (the CL group) did not significantly reduce wound strength, although the mean values of the strength measurements were lower than in the control group. This effect of 5-FU was independent of prior irradiation treatment: the decline in strength from C to CL and CH groups (Figure III-3, Table III-2) was similar to that from I to I/CL and I/CH groups. Thus the detrimental effect of 5-FU was not enhanced by preoperative irradiation.

In conclusion, our data show that the combination of preoperative irradiation and direct postoperative chemotherapy does not lead to detrimental effects on anastomotic strength, although (high-dose) chemotherapy alone significantly reduces anastomotic strength. However, the combined use of preoperative irradiation with high-dose postoperative 5-FU seems questionable because of increased frequency of rectal stenosis.

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COMBINED PREOPERATIVE IRRADIATION AND LOCAL HYPERTHERMIA DELAYS EARLY HEALING OF EXPERIMENTAL COLONIC ANASTOMOSES

J.Biert[*], W.F.Seifert[*], B.de Man[*], Th.Wobbes[*], J.Hoogenhout[#], Th.Hendriks[*]

From the Departments of [*]Surgery and [#]Radiotherapy, University Hospital Nijmegen, the Netherlands

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ABSTRACT

Purpose to determine if a combination of preoperative irradiation and local hyperthermia of a colonic segment is detrimental to subsequent early anastomotic healing
Methods and materials Eighty male Wistar rats were randomly divided into 4 groups In each animal, a segment of the colon was treated successively by (sham-)irradiation and (sham-)hyperthermia After 5 days a colonic resection was performed and an anastomosis was constructed the distal limb consisted of (sham-)irradiated, (sham-) hyperthermia-treated bowel The rats were killed 3 or 7 days after surgery The main outcome measures were body weight, serum albumin and protein levels, and anastomotic bursting pressure, breaking strength, and hydroxyproline content
Results All animals tolerated (sham-)treatment well Weight was diminished, though not notably, in treated animals vs the control group After combined preoperative irradiation and hyperthermia the frequency of local anastomotic complications increased 4 of 20 animals had a covered perforation when they were killed In this group the bursting pressure was lower 3 days after the operation (P=0 0078) The breaking strength was also lower, but not notably The serum albumin level was significantly lower in this group vs the control group (P=0 0056), the serum protein level was not decreased After 7 days no differences existed between groups The hydroxyproline content of the anastomotic tissue was significantly higher in rats treated with radiation plus hyperthermia vs control rats (in both the 3- and 7-day groups, P≤0 02) The anastomotic hydroxyproline concentration did not differ between groups
Conclusions The combination of preoperative irradiation and hyperthermia results in increased local anastomotic complications Anastomotic strength is at risk in the first days after the anastomotic reconstruction Preoperative irradiation or hyperthermia alone does not lead to impaired anastomotic healing in the early phase

INTRODUCTION

Regarding the surgical treatment of colorectal disease discussion continues about the role of adjuvant treatment modalities. Not all cancers can be cured by surgery alone. Along with distant metastases, the threat of a microscopic disease or a peri-operative tumor spill resulting in local recurrence exists. In addition some tumors have grown beyond local resectability before surgery can be performed. In the latter patients preoperative radiotherapy can help to obtain local control and to increase resectability (1) The application of hyperthermia is a promising method for increasing the efficacy of radiation therapy.
The biological effects of combined hyperthermia and radiotherapy are well established, although the mechanisms of interaction seem complex (2-5).

Enhanced treatment effects are to be expected in 2 ways (3-6). The first is the killing effect of heat itself on the hypoxic tumor cells. The second is the enhancement of the radiobiological effect of irradiation. Consequently lower radiation doses are needed to obtain the same treatment result. Thus, surrounding tissues that are located in the irradiated volume, will receive smaller doses of radiation, lessening the potential side effects. In patients with rectal cancer surgical treatment usually consists of an abdomino-perineal resection or a low anterior resection. In the latter cases, an anastomosis is constructed; after combined prior adjuvant treatment the fixed, distal limb has received the full dose of radiation plus heat treatment. Little is known about early anastomotic healing under these circumstances. Although the application of heat alone had no adverse effect on anastomotic repair in the rat ileum (7), no data about colonic healing exist. Also, data from our laboratory (8) and the laboratory of Weiber *et al.* (9) indicate that preoperative irradiation without detrimental effects on the early healing of colonic anastomoses is feasible. Still, no data are available on the effects of combined (preoperative) treatment.
This study was performed to develop an experimental model in the rat, and to investigate early colonic anastomotic healing after successive treatment of the anastomotic site with preoperative radiotherapy and hyperthermia.

METHODS AND MATERIALS

Animals
Eighty young adult male outbred Wistar/Cpb:WU rats (body weight 255g ± 5%) were used. They received water and standard laboratory food (diet AM II, Hope Farms, Woerden, The Netherlands) ad libitum. The rats were randomly divided into 4 groups of 20: a control group and 3 experimental groups which received irradiation (I), hyperthermic treatment (H) or both (I/H). In rats in the I and the I/H groups part of the colon was irradiated 5 days prior to the operation. Animals in the control and the H groups received sham irradiation, and those in the control and the I groups received sham hyperthermic treatment. All of the animals underwent colonic resection and anastomotic construction; they were killed 3 or 7 days (n=10 for both days) after surgery.

This study was approved by the Animal Ethics Review Committee of the Faculty of Medical Sciences, University of Nijmegen (the Netherlands).

Irradiation, dosimetry, and hyperthermia

To receive irradiation or hyperthermic treatment, the animals were anaesthetized with pentobarbital sodium intra-peritoneally. While receiving anaesthesia the core temperature of the animals was kept at a normal level by the use of a heat lamp.

The irradiation procedure was based on techniques developed in a prior experiment (8). To ascertain that the same tissue area was irradiated in each rat, and to mark this area for subsequent surgery, a laparotomy was performed. The colonic segment to be irradiated, which was 1 to 3.2 cm proximal to the recto-peritoneal fold (Figure IV-1), was marked by a serosal stitch at its proximal border. The irradiated area measured 2.2 x 0.5 cm^2. The adjacent bowel and other organs were covered with a lead cone and the rest of the body was also shielded with lead (thickness 2.5 mm). Radiation dosimetry was performed by means of thermoluminescent dosimeters and film densitometry in separate animals. Irradiation was performed with a 250 kV X-ray unit that had a 1 mm copper filter (target-colon distance 25 cm). The dose rate was 1.29 Gy/min. All of the rats in the I and the I/H groups received a dose of 25 Gy. Animals in the control and the H groups were treated similarly without actually being irradiated.

To deliver hyperthermic treatment, water washing through the distal colon and rectum was used (Figure IV-1). A small, non-injuring clamp was placed on the colon, at the level of the marking stitch, to isolate this bowel segment from the proximal colon, and a 1.75 mm plastic tube was gently inserted into the rectum up to the clamp. This tube was used to pump clean tap water through a coil, which was immersed in a temperature-regulated water bath into the rectal cavity (flow rate 132 ml/min), while a small intrarectal temperature probe was used to monitor the intra-luminal temperature. A second 1.75 mm tube served as a drain, preventing increased pressure inside the rectal cavity. The infusion of water into the rectum was continued for 30 minutes, while the intra-rectal temperature was kept at 44°C (this temperature was usually reached within 3 minutes [H and I/H groups]). The intra-rectal temperature quickly returned to normal after the heating was discontinued. Subsequently, the clamp was removed from the bowel, and

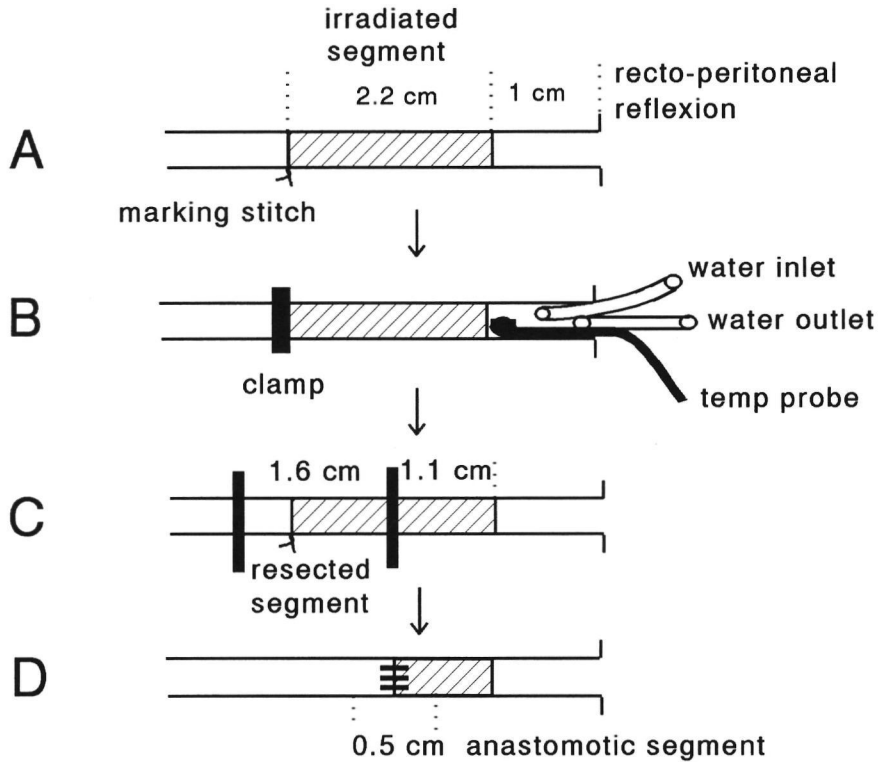

Figure IV-1. A schematic of the experimental procedure. **A.** Irradiation of the colonic segment. **B.** The hyperthermia procedure. A clamp is placed at the proximal border of the irradiated segment. The inflow tube, the outflow tube and the temperature probe are placed in the rectum. **C.** Resection of the colonic segment and construction of anastomosis with a distal, irradiated limb. **D.** The sample used for hydroxyproline analysis.

the abdomen was closed with a running catgut suture for the fascia and staples for the skin. Therefore, the animals in the H and the I/H groups received hyperthermic treatment from 10 minutes after the conclusion of the (sham-)irradiation procedure onward. The animals in the control and the I groups were treated similarly with water of a normal body temperature (37°C).

Operative procedure

After 5 days, the animals were anaesthetized again intra-peritoneally with pentobarbital sodium. The median laparotomy wound was opened and a 1.6 cm colonic segment was resected. This segment was identified by the marking stitch left during the initial laparotomy and measured 0.5 cm

proximally and 1.1 cm distally (Figure IV-1). Thus, the proximal limb to be used for the anastomosis consisted of non-irradiated, unheated tissue, while the first 1.1 cm of the distal limb had been irradiated (ie, for the I group), heated (ie, for the H group) or both (ie, for the I/H group). Continuity was restored by an inverted 1-layer end-to-end anastomosis with 8 interrupted monofilament sutures (Ethilon 8-0, Ethicon®, Norderstedt, Germany) using microsurgical techniques. The fascia and the skin were closed with a catgut running suture and staples, respectively.

Analytical procedures

The condition of the animals was monitored, and their weight was measured daily. Three or 7 days after the operation the rats were killed by cardiac puncture. A blood sample was obtained to measure albumin and protein levels to monitor the general nutritional condition of the animals. The abdomen was inspected for adhesions, rectal stenosis, abcesses or other abnormalities. The anastomoses were resected en bloc. Healing was assessed by measuring the anastomotic strength and the hydroxyproline content (10). To determine the anastomotic strength, the anastomotic segment was washed in a saline solution and connected to an infusion pump on 1 side while the other side was clamped. A manometer was connected by a side line. The bursting pressure was measured by raising the intra-luminal pressure by infusing a methylene blue-saline solution at a rate of 2 ml/min. The procedure was performed in water for better visualization of the bursting site. The bursting pressure was defined as the maximal intra-luminal pressure the segment resisted, which was expressed in millimeters of mercury. The bursting site was noted. The breaking strength of the segment, as a measure of the resistance to longitudinal forces, was measured immediately after determination of the bursting pressure (11). The segment was placed in a tensiometer that provided an constantly increasing distraction. The peak force (in gram) necessary to induce the total disruption of the segment was perceived as the breaking strength. The breaking site was noted. Then, adhesions and fat tissue were removed from the segment, and a 0.5 cm sample containing both sides of the suture line was collected (Figure IV-1), stored in liquid nitrogen, and assayed for hydroxyproline content.

Anastomotic samples were weighed, lyophilized, pulverized, and stored at -30° C. The hydroxyproline content, as a measure of collagen content, was measured by highperformance liquid chromatography after hydrolysis with 6N hydrochloride and derivatization with dabsylchloride.
Differences between the control group and the 3 experimental groups were tested for significance (P=0.033) using a 2-tailed Mann-Whitney U test. To correct for the fact that multiple comparisons were made, pairwise comparisons were performed using a level of significance of $\alpha^{I}=2\alpha/k$, where k is the total number of pairwise comparisons. Thus, differences between groups were considered significant (α=0,05) at $P<\alpha^{I}$, where α^{I} = 0,033.

RESULTS

General observations
All of the animals tolerated hyperthermic treatment, radiotherapy, or both well. Four rats died before they were killed, 1 in each of the 4 groups; the time of death was different in all 4 animals and was thought to be anaesthesia-related; at autopsy no intra-abdominal causes of death were found.
Rats receiving either hyperthermia or a radiation dose had moderate adhaesions at the time of resection. The bowel wall increased in diameter because of edema, and it was more vulnerable in these animals vs. the sham-treated animals. These rats also suffered from minimal to moderate diarrhoea.
In rats that received both treatments, some gross abnormalities were found. Six rats in the I/H group had small patches of transmural bowelwall necrosis immediately distal from the marking stitch in the control segment that was removed during surgery. Four rats had anastomotic leakage, which was apparent by a covered perforation when they were killed.
Small abcesses around the anastomoses were occasionally seen in all groups: the C group, 2; the I group, 3; the H group, 1; and the I/H group, 2. Rectal stasis or functional stenosis with a proximal dilated colon was seen in 2 rats, 1 each in the I and the H groups.

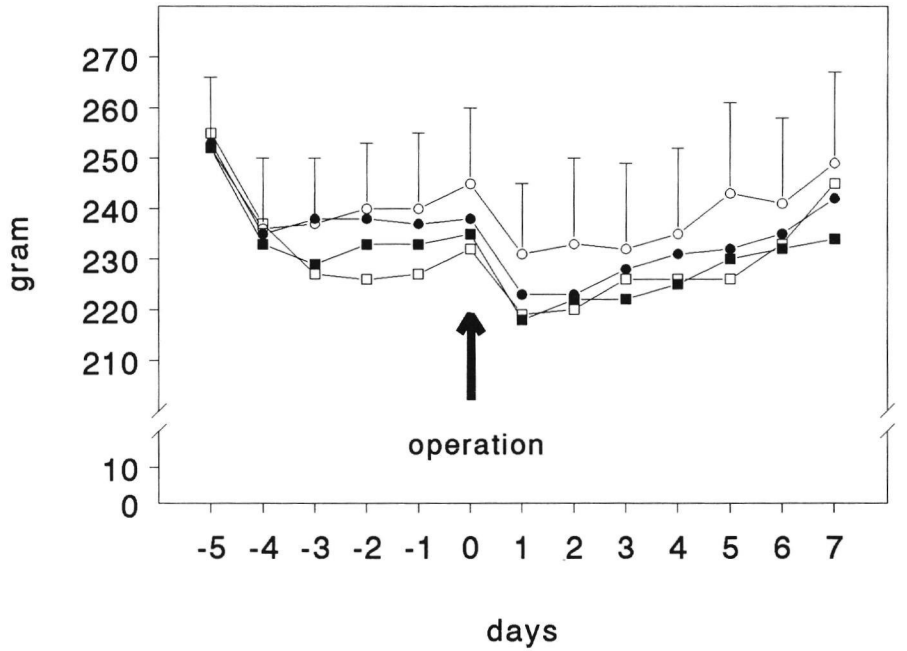

Figure IV-2. Weight changes in the 7-days groups during the experiment. Open circels (+ SD): C group (control animals); open squares: H group (hyperthermic treatment); closed circels: I group (irradiation); closed squares: I/H group (irradiation and hyperthermic treatment).

Body weight

After receiving the first dose of anaesthesia, all of the rats lost weight. Hyperthermia-treated animals (ie, the H and the I/H groups) seemed to lose more weight than those in the other groups (Figure IV-2). However, recovery seemed to be similar in all groups from postoperative day 2 onwards. Colonic surgery induced a transient loss of body weight, but the size of this effect and the subsequent course of recovery were similar in all groups. No notable differences in body weight were found between groups on the day that the rats were killed.

Serum albumin and protein

The levels of serum albumin and protein are given in the Table IV-1. Three days after the operation, the serum albumin level was slightly but significantly (P=0.0056) lower in the I/H group. This difference was not found after 7 days. The only significant (P=0.01) reduction in serum protein levels was found in the I group after 7 days.

Anastomotic strength

Individual measurements of bursting pressure and bursting site are depicted in Figure IV-3. In all groups, the bursting strength increased between days 3 and 7 after the operation. The average bursting pressure in the I/H group was significantly (P=0.0078) reduced vs. the control group 3 days after the operation. In addition, the bursting site was more often within the suture line in the treatment groups vs. the control group, particularly in the I/H group Seven days after the operation, anastomoses usually ruptured outside the anastomotic area, indicating that anastomotic healing had progressed beyond the values of normal bowel wall resistence. The average bursting pressures in the experimental groups did not differ notably from that in the control group.

The mean values for the anastomotic breaking strength are depicted in Figure IV-4. The breaking strength, like the bursting pressure, increased from day 3 to day 7.

Three days after the surgery, the values were slightly lower in the I/H group vs. the control group. The breaking site was inside the anastomosis in all groups except for 1 rat in the I group.

No notable differences in breaking strength were observed between experimental and control groups 7 days after the operation. The breaking site was within the suture line in 6 of 10 cases in the control group and in 2 of 9, 3 of 9, and 3 of 10 cases in the H, the I and the I/H groups, respectively

Table IV-1. *Serum albumin and protein (mean ± SD, n=9 or 10) at day of sacrifice*

	serum albumin *(g/l)*		serum protein *(g/l)*	
	3 days	7 days	3 days	7 days
group C	22 6 ± 0 9	22 8 ± 1 0	55 7 ± 0 9	57 3 ± 2 0
group H	22 8 ± 1 0	22 2 ± 0 7	56 2 ± 2 0	57 6 ± 2 1
group I	21 9 ± 1 2	22 0 ± 1 0	55 3 ± 2 7	54 0 ± 2 8*
group I/H	21 1 ± 0 9*	23 5 ± 1 4	54 2 ± 3 2	57 8 ± 3 0

* P<0 033 (Mann-Whitney U test) difference vs control group

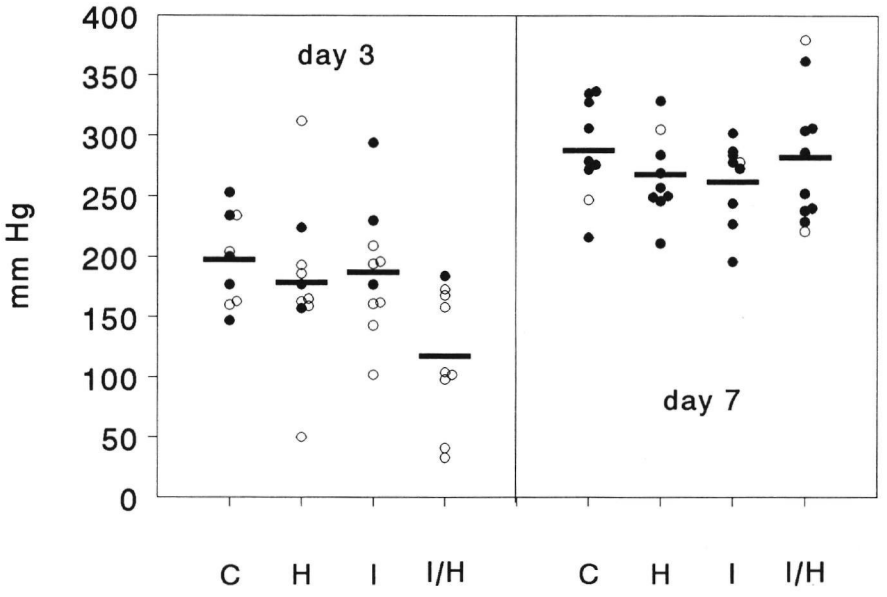

Figure IV-3. Average anastomotic bursting pressure. Each point represents a measurement in 1 animal. C group: control animals; H group: hyperthermic treatment; I group: irradiation; I/H group: irradiation and hyperthermic treatment. Open circels: bursting site within suture line; closed circels: bursting site outside suture line.

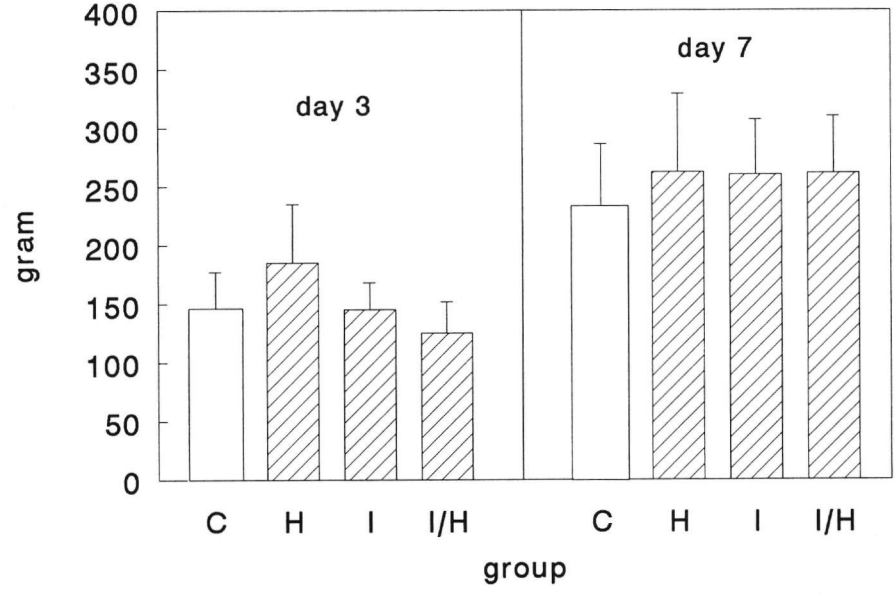

Figure IV-4. Anastomotic breaking strength. Bars represent average values + SD (n=9 or n=10). The groups are described in the legend for Figure IV-3.

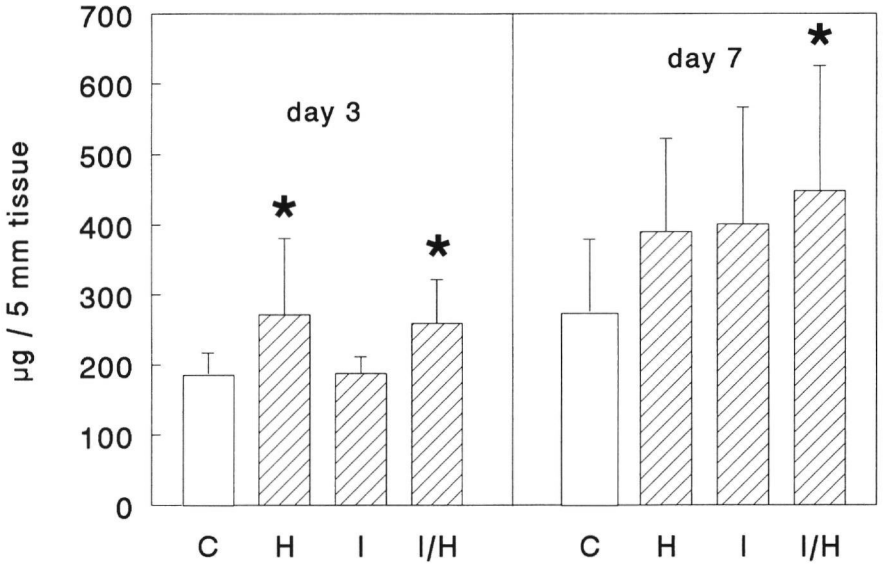

Figure IV-5. Hydroxyproline content of anastomotic segments. Bars represent average values + SD (n=9 or n=10). The groups are described in the legend for Figure IV-3.
*: P≤0.033 (2-tailed Mann-Whitney U test) difference vs. the control group.

Anastomotic hydroxyproline

The hydroxyproline content of the anastomotic segments increased between days 3 and 7 after the operation (Figure IV-5). On day 3, the anastomotic hydroxyproline content was significantly higher in the H (P=0,022) and the I/H (P=0,003) groups vs. the control group. On day 7, only the hydroxyproline content of the I/H group was significantly (P=0,018) elevated.

The average hydroxyproline concentrations in the anastomotic segments from the control group were 10.1 ± 1.0 µg/mg of dry weight on day 3 and 13.4 ± 1.4 µg/mg of dry weight on day 7. The values found in the experimental groups were not notably different.

DISCUSSION

This study shows that high dose preoperative irradiation of the colon with a dose of 25 Gy, combined with local hyperthermia (44°C, 30 minutes), results in increased local anastomotic complications. The high incidence of

covered perforations in the I/H group suggests that local reaction is such that late anastomotic problems, like stenosis, can be expected as well. In addition, the data demonstrate that in this model neither local hyperthermia nor irradiation alone has an adverse effect on subsequent anastomotic healing. The fact that hyperthermia increases the biological effects of irradiation (2-6) may explain the finding that a combination of 2 tolerated treatment modalities results in serious complications.

Hyperthermia seems to improve the tumor-killing effects of radiotherapy in 2 ways. First, hyperthermia directly affects the cells. Hypoxic, poorly nourished cells in an environment with a relatively low pH and poor perfusion are succeptible to hyperthermic damage. These cells are often found in tumors, and they are usually relatively radioresistant. The killing of tumor cells by heat is established within 24 hours after application, and is caused by damage to membrane lipoproteins and by denaturation of thermolabile cellular enzymes (3,6). Second, hyperthermia has a radiosensitizing effect. This effect can only be expected if hyperthermia preceeds irradiation (3), and it seems to be caused by inhibition of sublethal damage repair and blockage of cell proliferation (6,12). The combined effect of radiation therapy and hyperthermia as an adjuvant treatment for surgery seems to be promising; although only reports of incidents or small series are available, the outlook for patients with locally irresectable colorectal cancer seems to be favorable (13-22). However, numerous questions need to be answered if hyperthermia is considered for wide-spread clinical use.

Discussion continues about the optimal timing and sequence for the combination of radiotherapy and hyperthermia. The sequence where hyperthermia precedes irradiation has been advocated as the most effective, primarily because of a radiosensitizing effect (5). However, radiotherapy followed by hyperthermia has also been proposed to be more effective; using this sequence, the increased effect is mainly owing to the direct heat killing of radioresistant cells (3,18,20,23-26). Because it has also been noted that human colon cancer cells show evidence of radioresistance (20), the latter sequence might be more appropriate for patients with colorectal cancer.

Because radiotherapy in patients with colorectal cancer is often given preoperatively, we have chosen to investigate the potential detrimental effects of preoperative irradiation followed by hyperthermia on anastomotic

healing in the colon. Hyperthermia was applied immediately after radiotherapy because this method has been reported to yield a maximal effect on tumor cells and less of an effect on normal cells (25). A temperture of 44° C for 30 minutes seems to yield the maximal effects, but is still safe (7). A single application was chosen to prevent the influence of factors like fractionated treatment, thermotolerance (ie, the phenomenon that a second dose of hyperthermia is less effective), and step-down heating (ie, increased effectiveness if maximal hyperthermia is immediately followed by a period of mild hyperthermia) (3,5,6). A 30 minute treatment is biologically possible in animal experiments, and is derived from other experimental work (7,27).

Three days after operation, the anastomotic bursting pressure was clearly and significantly ($P=0.0078$) reduced in the I/H group, while the breaking strength was only marginally affected. Early anastomotic strength is mainly determined by the capacity of the submucosal collagen network to retain the sutures. A localized loosening of this structure, by the enhanced activity of collagenolytic enzymes, may lead to easier local rupture at inflation (and lowered bursting pressure) while the breaking strength remains largely unaffected. Whether local degradation of anastomotic collagen occurs within the I/H group still needs to be established. The existence of such a process cannot be derived from the current measurement of the anastomotic collagen (as hydroxyproline) content, which is too insensitive to demonstrate the localized loss of collagen.

The observation that the hydroxyproline content of the anastomotic segment, which contains normal bowel wall next to the actual wound area, is actually significantly ($P \leq 0.022$) increased in the I/H group, indicates the occurrence of a strong fibrotic reaction; if this reaction persists, late complications may also be expected in this group.

Preoperative protein malnutrition may affect anastomotic strength (28). Three days after the operation, the average serum albumin and serum protein levels were 7% and 3% lower, respectively, in the I/H group vs. the control group. However, it seems unlikely that the colonic anastomotic strength is affected by this mild hypo-albuminemia.

No studies about the effects of hyperthermia, alone or in combination with radiotherapy, on anastomotic healing have been conducted. The influence of hyperthermia on small bowel anastomoses was described in 1 study (7).

Hyperthermia was applied intra-peritoneally to study the prevention of serosal metastases in patients who underwent gastric cancer surgery. Survival decreased when temperatures of 45°C or higher were used. Local hyperthermia for 30 minutes at 44°C showed no adverse effect on anastomotic healing in the ileum (7), although an increased adhaesion formation might occur (29,30). Our data confirm this result for anastomoses in the colon.

Experiments about anastomotic healing after preoperative radiotherapy have been described in various articles. This adjunct treatment was long considered to be detrimental for anastomotic healing (31-33). However, Biert *et al.* (8) and Weiber *et al.* (9) have shown that preoperative irradiation without negative effects on anastomotic healing is feasible, depending on factors like total dose, irradiation technique, number of fractions and irradiated volume (1). We confirmed this belief; irradiated, normotherm rats (ie, the I group) had anastomotic healing similar to the control group.

The results from our treatment protocol raise additional questions. Is the combination of radiotherapy and hyperthermia safe if a smaller radiation dose is used, or if the temperature or duration of the hyperthermia treatment is reduced? Are there any late side effects? More experimental work is needed to answer these and other questions before application of this combined modality can be considered for clinical use. We believe that this model is suitable to conduct such investigations.

STATEMENT OF CLINICAL RELEVANCE

In the treatment of colorectal cancer, surgical results show that adjuvant therapy is neccessary. Adjuvant radiation therapy is generally accepted as a method for providing an improvemened outcome when used in the preoperative setting. The search continues for new treatment modalities to prevent local recurrence and distant metastasis. Few clinical trials in which hyperthermia was used as an addition to irradiation have been published. The first results are promising. A low anterior resection is performed in cases; the anastomotic reconstruction is performed with a proximal, untreated limb, and a distal limb that has been irradiated and treated with heat. Anastomotic healing under these circumstances is at risk of dehiscense.

We investigated the possible side effects of this adjuvant treatment on anastomotic repair in an animal experiment. Although anastomotic strength was not decreased, there seemed to be increased local anastomotic complications, posing a risk for early anastomotic repair in the first days after the operation.
The results show that further studies about the use of the combination of preoperative irradiation and hyperthermia are needed to provide more insight about the mechanisms of anastomotic wound healing under this condition.

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Chapter 5

A SEMI-QUANTITATIVE HISTOLOGICAL ANALYSIS OF ANASTOMOTIC WOUND REPAIR IN THE RAT COLON AFTER COMBINED PREOPERATIVE IRRADIATION AND LOCAL HYPERTHERMIA

J.Biert[*], W.F.Seifert[*], A.A.J.Verhofstad[§], Th.Wobbes[*], B.M.de Man[*], J.Hoogenhout[#], Th.Hendriks[*]

From the Departments of [*]Surgery, [§]Pathology and [#]Radiotherapy, University Hospital Nijmegen, the Netherlands

submitted

ABSTRACT

Purpose. to study the microscopical aspects of colon anastomotic healing after combined preoperative (sham-)irradiation and (sham-)hyperthermia treatment.
Methods and materials: 48 male Wistar rats were randomly divided into four groups. In each animal, a segment of the colon was treated successively by (sham-)irradiation (single dose of 25 Gy) and/or (sham-)hyperthermia (44°C, 30 minutes). After 5 days a colonic resection was performed with construction of an anastomosis: the distal limb consisted of (sham-)irradiated and/or (sham-)hyperthermia-treated bowel. Rats were sacrificed 3 or 7 days after surgery. Evaluation of anastomotic healing was made by: 1. histological parameters on Haematoxylin-Eosin stained sections; 2. semi-quantitative measurement of collagen in the anastomotic area; 3. semi-quantitative analysis of the number of macrophages by immunocytochemistry.
Results: anastomotic healing in animals receiving irradiation or hyperthermia alone, and in controls was relatively uneventful. There were no differences in collagen formation or macrophage infiltration in the anastomotic area between groups. Animals treated with both irradiation and hyperthermia showed marked necrosis, infiltration by polymorphonuclear leucocytes, and anastomotic dehiscence.
Conclusions: preoperative irradiation of a colonic segment with a single dose of 25 Gy in combination with local hyperthermia of 44°C for 30 minutes leads to disturbed anastomotic repair.

INTRODUCTION

Application of hyperthermia is a method to increase the efficacy of radiation therapy. The biological effects of combined hyperthermia and radiotherapy are well established, although the mechanisms of interaction appear complex (1-4). Enhanced treatment effects are caused by the direct killing effect of both modalties, and by interfering with the radiobiological processes, such as repair of subeffective damage (2-5). As a consequence, with combined treatment lower radiation doses can be used to obtain the same treatment result. Thus, surrounding tissues in the irradiated volume will receive smaller doses of radiation, lessening potential radiation side ef fects. Very recently we have studied anastomotic strength following combined preoperative irradiation and hyperthermia in rats (6). The fixed, distal limb of the anastomosis received 25 Gy followed by 30 minutes heat treatment with water of 44°C, while the proximal limb was composed of untreated tissue. As a consequence local anastomotic wound problems were enhanced, although early anastomotic strength was not significantly reduced.

With regard to the application of heat alone, it has been reported that there is no adverse effect on anastomotic repair in the rat colon (6) or ileum (7). Also, recent data from our own laboratory (6,8) and others (9) indicate that preoperative irradiation without detrimental effects on early healing of colonic anastomoses is possible. So far, no comprehensive histological evaluation has been reported of early healing in intestinal anastomoses constructed after irradiation and/or hyperthermia. The aim of the present study was to examine the histological changes during the early healing phase, and to (semi-quantitatively) measure these changes.

METHODS AND MATERIALS

Animals

Forty-eight young adult male outbred Wistar/Cpb:WU rats, body weight 255 g ± 5%, were used. They received water and standard laboratory food (diet AM II, Hope Farms, Woerden, The Netherlands) ad libitum. The rats were randomly divided into 4 groups of 12: a control group (C) and 3 experimental groups which were irradiated (I), treated with hyperthermia (H) or both (I/H). In rats in the I and I/H groups a part of the sigmoid colon was irradiated 5 days prior to operation. Animals in groups C and H were sham-irradiated. In addition animals in the H and I/H groups received hyperthermic treatment, while animals in the C and I groups received sham-hyperthermic treatment. All animals underwent colonic resection and anastomotic construction; they were sacrificed 3 days (n=6) or 7 days (n=6) after surgery.

This study was approved by the Animal Ethics Review Committee of the Faculty of Medical Sciences, University of Nijmegen.

Irradiation, dosimetry, and hyperthermia

During the irradiation or the hyperthermia treatment, the animals were anaesthetized with intraperitoneal sodium pentobarbital.

The irradiation procedure was based on techniques developed in a prior experiment (8). To ascertain that the same tissue area was irradiated in each rat, and to mark this area for subsequent surgery, a laparotomy was performed. The colonic segment to be irradiated, 1 - 3.2 cm proximal from

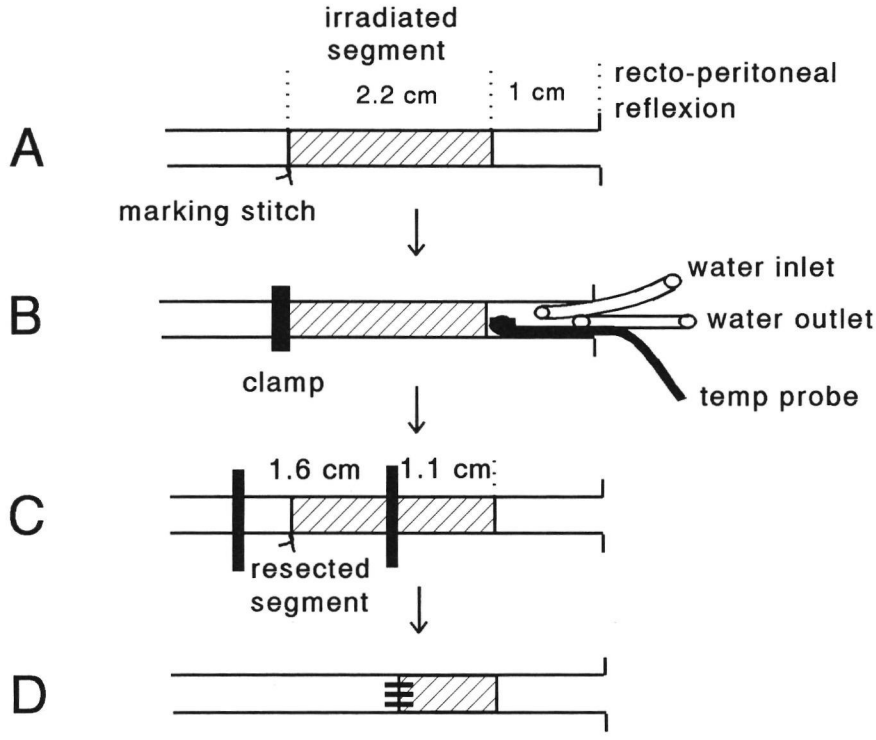


Figure V-1. Schematic representation of the different steps in the experimental procedure. **A**. Irradiation of colonic segment. **B**. Hyperthermia procedure. A clamp is placed at the proximal border of the irradiated segment. Inflow tube, outflow tube and temperature probe are put in the rectum. **C**. Resection of colonic segment and construction of anastomosis with a distal, irradiated limb. **D**. Situation at sacrifice.

the recto-peritoneal fold (Figure V-1), was marked by a serosal stitch at its proximal border. The irradiated area measured 2.2 x 0.5 cm^2. The adjacent bowel and other organs were covered with a lead cone and the rest of the body was also shielded with lead (thickness 2.5 mm). Radiation dosimetry was performed by means of thermoluminescent dosimeters and film densitometry in separate animals. Irradiation was performed with a 250 kV X-ray unit with a 1 mm Cu filter (target-colon distance 25 cm). The dose rate was 1.29 Gy/min. All rats in the I and I/H groups received a single dose of 25 Gy. Animals in the C and H groups were treated similarly without actually being irradiated.

Hyperthermia was delivered by washing water through the distal colon and rectum (Figure V-1), according to a technique developed in a prior experiment (6). For this purpose a small, non-injuring clamp was put on the

colon, at the level of the marking stitch, to isolate this bowel segment from the proximal colon, and a 1.75 mm diameter plastic tube was gently inserted into the rectum up to the clamp. This tube was used to infuse tap water (flow:132 ml/min; temperature 37° or 44° C), while a small intrarectal temperature probe was used to monitor the intraluminal temperature. A third 1.75 mm diameter tube served as a drain, preventing increased pressure inside the rectal cavity. Infusion of the rectum was continued for 30 min, while the intra-rectal temperature was kept at 44°C, which temperature was usually reached within 3 min (H and I/H groups). The intrarectal temperature quickly returned to normal values after the heating was discontinued. Subsequently, the bowel clamp was removed and the abdomen was closed with a running catgut suture for the fascia, and staples for the skin. In this way, animals in the H and I/H groups received hyperthermia from 10 min after conclusion of the (sham-) irradiation onwards. Animals in the C and I groups were treated similarly with water of normal body temperature (37°C).

Operative procedure

After 5 days the animals were anaesthetized again with intraperitoneal sodium pentobarbital. The median laparotomy wound was opened and a 1.6 cm colonic segment was resected. This segment was identified by the marking stitch left during the initial laparotomy; the length of the segment measured 0.5 cm in proximal and 1.1 cm in distal direction (Figure V-1). Thus, the proximal limb to be used for the anastomosis consisted of non-irradiated, non-heated tissue, while the first 1.1 cm of the distal limb had been irradiated (I and I/H groups) and/or heated (H and I/H groups). Continuity was restored by an inverting one-layer end-to-end anastomosis with 8 interrupted monofilament sutures (Ethilon 8-0, Ethicon®, Norderstedt, Germany) using microsurgical techniques. Fascia and skin were closed with a catgut running suture and staples, respectively.

Analytical procedures

Three or seven days after operation the rats were sacrificed by cardiac puncture. The abdomen was inspected for adhesions, stenoses, abcesses or other abnormalities. The anastomoses were resected en bloc, and the anastomotic segment was washed gently in saline and cut longitudinally in

2 equal parts. One part was fixed in 4% phosphate buffered formaldehyde, dehydrated and embedded in paraffin. Parrafin sections of 4 μm in thickness were stained with haematoxylin-eosin (HE) for histological evaluation. Sirius Red staining, as modified by Junqueira *et al.* (10) was used to demonstrate collagen. The other part was immediately deep frozen in liquid nitrogen. Cryostat sections of the frozen material, 4 μm in thickness, were first fixed in acetone for 10 min at 4°C and stained by the indirect immunoperoxydase technique, using a monoclonal antibody to rat macrophages (clone ED3; Serotec, Oxford, U.K.). The sections were studied by one of the investigators who was unaware of the kind of treatment that the animal had undergone.

In the HE-stained sections, the integrity of the anastomosis was assessed. For this purpose, it was decided if the anastomosis was intact (i.e. if the epithelium was closed), and if there was massive cellular infiltration in tissue surrounding the anastomosis, which would suggest disturbed healing. Also, it was examined if the muscularis layer was healed, adjacent, or separated in the anastomotic area. As a consequence of these observations, healing of each individual anastomosis was pronounced to be normal, partially disturbed or completely disturbed.

Using the Sirius Red stained sections, a semiquantative measurement was performed of the amount of collagen in the anastomotic region. The anastomotic region was defined as a row of adjacent viewfields (magnification 100x) on either side of the anastomotic line. For this purpose, collagen fibers were counted with the use of a special count-ocular. This ocular was equipped with a grid of 42 measure spots. The presence of collagen fibers on these spots were considered to be a positive result. The percentage of positive results was determined in all adjacent view-fields of the anastomotic region. The differences between experimental and control groups were tested for significance using a two-tailed Mann-Withney-U test. To correct for the fact that multiple comparisons were made, pairwise comparisons were performed using a level of significance of $\alpha'=2\alpha/k$, were k is the number of pairwise comparisons. Thus, differences between groups were considered significant ($\alpha=0.05$) at $P<\alpha'$, where $\alpha'=0.03$.

In the HE-stained sections, the anastomotic region was examined for polymorphonuclear (PMN) cells, lymphocytes, and necrosis. Measurements were performed semi-quantatively;

possible scores were:

0: normal amount of cells,
1: little increase of amount of PMN cells,
2: marked infiltration with PMN cells,
3: massive infiltration with PMN cells.

0: normal amount of cells,
1: little increase of amount lymphocytes,
2: marked infiltration with lymphocytes,
3: massive infiltration with lymphocytes.

0: no necrosis,
1: one small patch of necrosis,
2: several patches of necrosis,
3: massive necrosis.

The number of macrophages as revealed by immunoperoxydase staining was also estimated semiquantatively, and possible scores were:

0: no cells found,
1: isolated cells,
2: more cells,
3: cells in clusters,
4: massive reaction.

RESULTS

General observations and macroscopical inspection after sacrifice
Three animals died, one in the I/H group and two in the H group. The one death in the I/H group was due to anastomotic dehiscence. The other deaths were caused by depression of ventilation due to the anaesthesia. Obduction revealed no evidence for dehiscence. Adhesions were seen in all groups, especially in groups treated with hyperthermia.
At the time of operation, two rats out of 12 in the I/H group showed marked

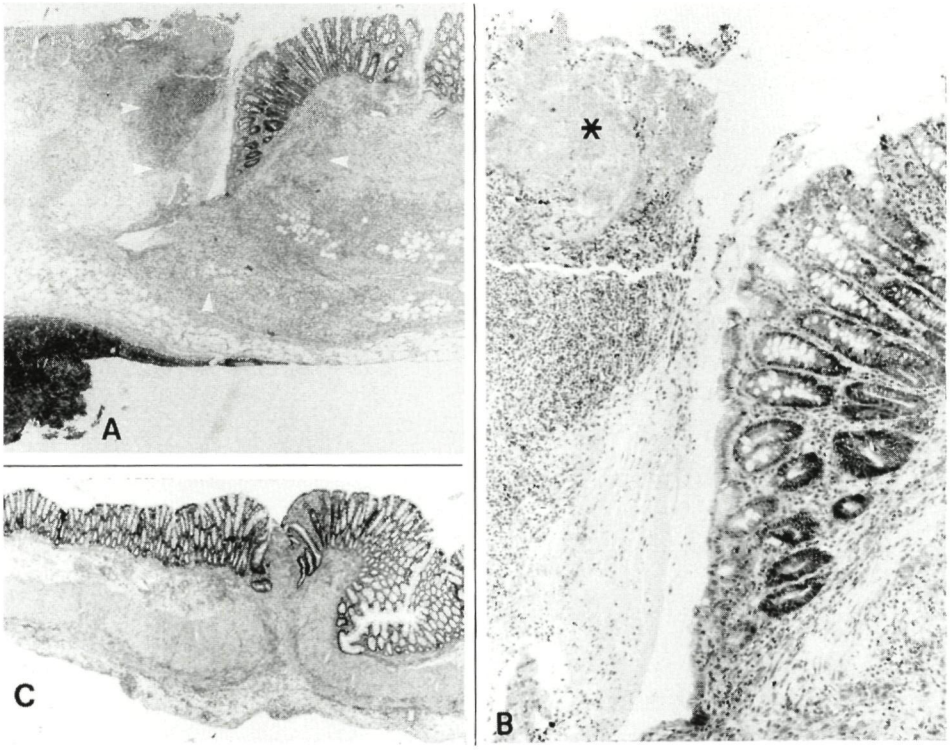


Figure V-2 A,B,C. Photomicrograph (**A**) and detail (**B**) of a HE-stained slide of a rat treated with radiation and hyperthermia (I/H group; the histological score is 3) 7 days postoperatively showing thickening of the bowel wall, polymorphonuclear infiltration (arrows), and necrosis (asterisk). Left side: absence of epithelium (acute radiation effect). **C**: comparable control rat (C group; the histological score is 1).

transmural necrosis in the resected colonic segment. One of these rats showed an ileus at the time of sacrifice (7 days) with distension of the proximal colon of 1 cm. In the same group two others developed anastomotic dehiscence.

One animal in the H group showed considerable anastomotic abcess formation.

Microscopical results

1. Anastomotic healing

Three days after the operation, anastomotic healing was completely disturbed in 3 out of 6 rats in the I/H group, in 1 out of 5 in the I group, in 1 out of 4 in the H group, and in 1 out of 6 in the C group. Partially

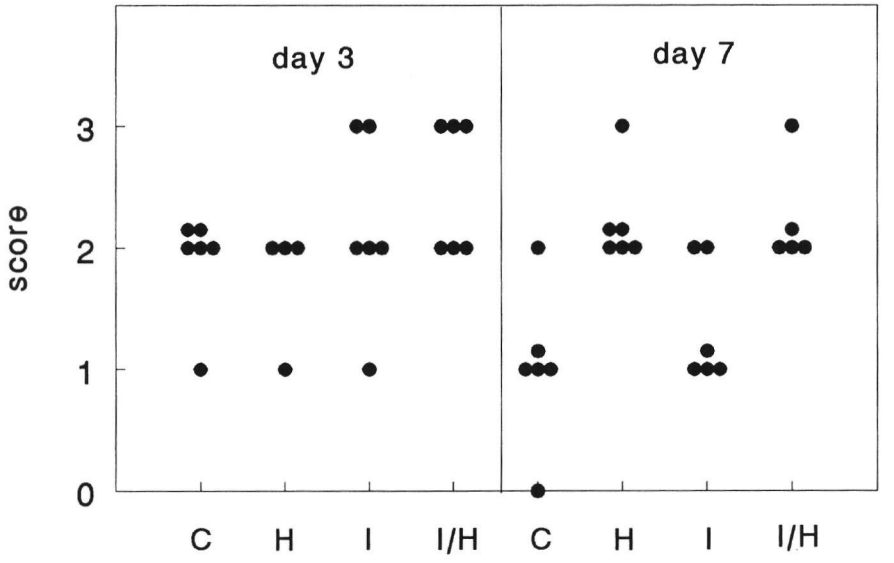


Figure V-3. Granulocyte infiltration on day 3 and day 7. C group: sham-treated animals. H group: sham-irradiation, hyperthermia. I group: irradiation, normal temperature. I/H group: irradiation, hyperthermia. Each point represents the findings in one animal.

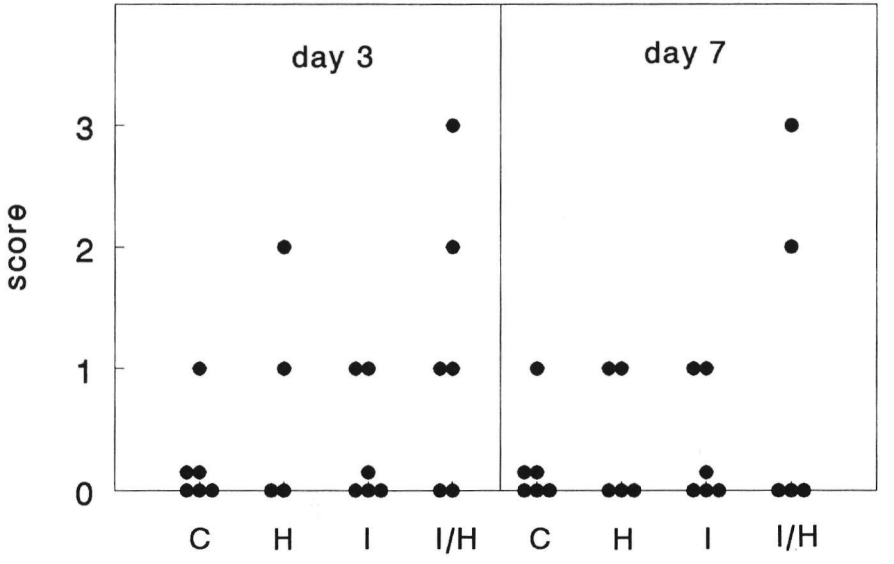


Figure V-4. Lymphocyte infiltration on day 3 and day 7. C group: sham-treated animals. H group: sham-irradiation, hyperthermia. I group: irradiation, normal temperature. I/H group: irradiation, hyperthermia. Each point represents the findings in one animal.

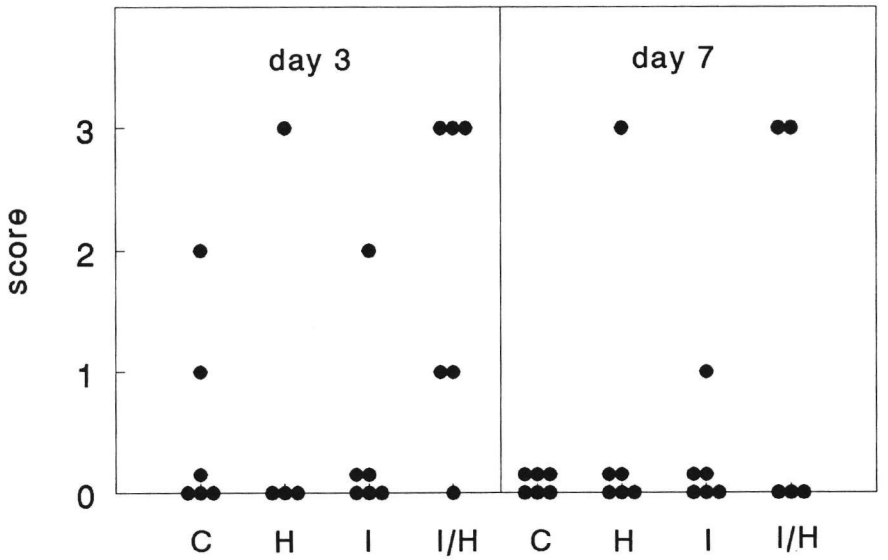
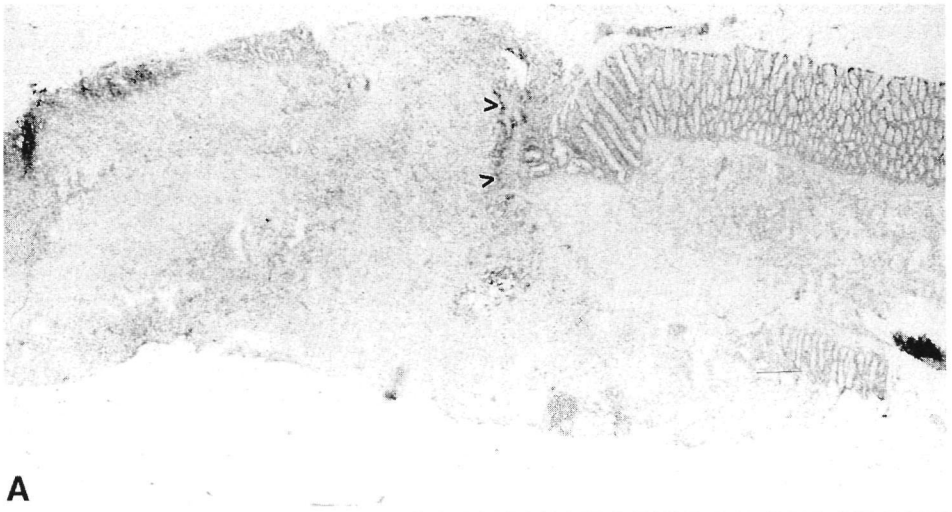


Figure V-5. Necrosis on day 3 and day 7. C group: sham-treated animals. H group: sham-irradiation, hyperthermia. I group: irradiation, normal temperature. I/H group: irradiation, hyperthermia. Each point represents the findings in one animal.

disturbed healing was present in 2 out of 6 rats in the I/H group.
Seven days after the operation, anastomotic healing was completely disturbed in 2 out of 5 rats in the I/H group.

2. Cellular infiltration, necrosis

In general, a moderate amount of polymorphonuclear cells was seen (after HE-staining) in the anastomotic area 3 days after the operation (Figures V-2 and V-3). At 7 days, these cells were much more dominant in the I/H and H groups than in the control group. Lymphocytes were sparse, and there appeared to be no systematic difference between groups, although 4 animals in the I/H group and 1 in the H group showed a lymphocyte infiltrative response (Figure V-4).
Necrosis was present to a certain degree in all groups; it was most pronounced in group I/H, both 3 and 7 days after operation. Necrosis did not occur in the C group 7 days after the operation (Figure V-5).
After irradiation, the distal limb of the anastomosis showed edema in the submucosa with superficial mucosal necrosis. In the group treated with hyperthermia alone the distal limb showed a slight polymorphonuclear infiltrate, without necrosis of the mucosa.



A

B

Figure V-6 A,B. Overview (**A**) and detail (**B**) of positive staining of macrophages (arrows) by ED3 in a rat treated with radiation and hyperthermia (I/H group; the histological score is 3) 7 days postoperatively.

Macrophage infiltration was clearly visible using the ED3 staining technique, on frozen sections (Figure V-6). In 3 rats, the quality of staining proved too poor to allow evaluation. In general, there seemed to be no overt differences between groups with regard to macrophage infiltration (Figure V-7).

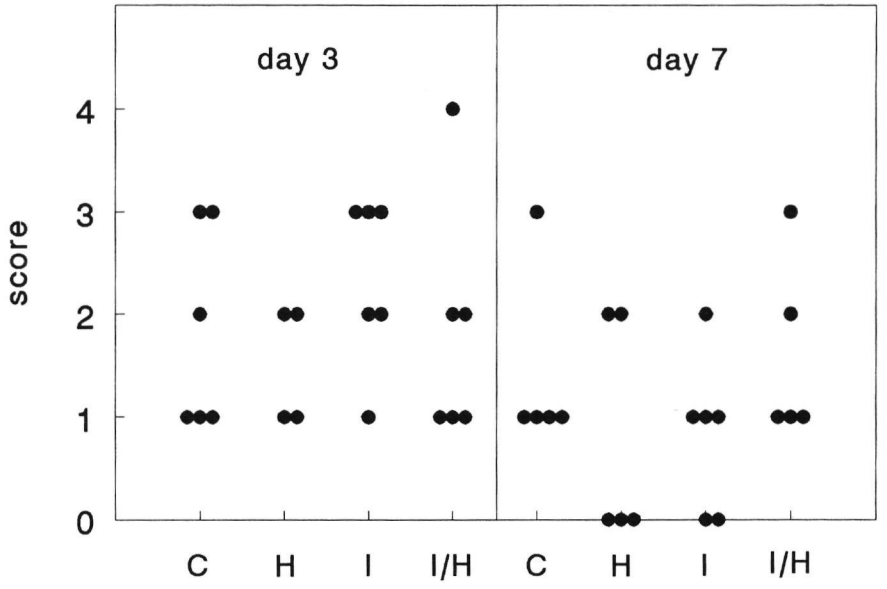


Figure V-7. Semiquantative, peri-anastomotic counts of macrophages (ED3). C group: sham-treated animals. H group: sham-irradiation, hyperthermia. I group: irradiation, normal temperature. I/H group: irradiation, hyperthermia. Each point represents the findings in one animal.

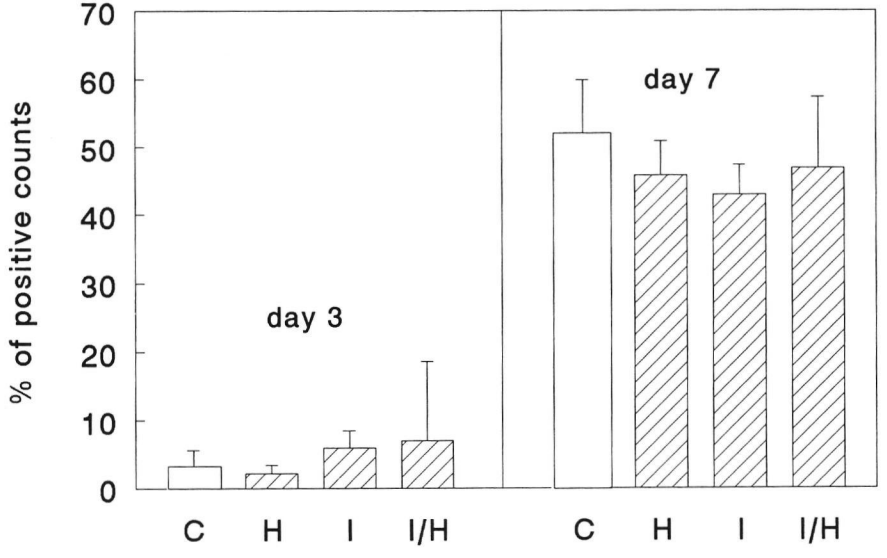


Figure V-8. Semiquantative, perianastomotic collagen fiber count. C group: sham-treated animals. H group: sham-irradiation, hyperthermia. I group: irradiation, normal temperature. I/H group: irradiation, hyperthermia.

3. Collagen measurement
The percentage of positive counts in the various groups is shown in Figure V-8. The presence of collagen was measured at 248 measuring points in 5.9 view-fields (average) in each section. However, in some rats the amount of anastomotic tissue was so small that only 4 adjacent view-fields with 168 measuring points were available.
In general, little collagen was present in the anastomotic region 3 days after operation. After 7 days, the amount of collagen, expressed as the number of positive counts, had increased markedly; this was similar in all groups. There were no statistically significant differences between the experimental and the control groups, neither 3 nor 7 days after the operation.

DISCUSSION

In this study colonic anastomotic healing after combined preoperative irradiation and hyperthermia was assessed histologically. A previous study had revealed that combined irradiation and hyperthermic treatment was followed by increased incidence of macroscopic bowel wall necrosis, leading to loss of anastomotic strength, whereas irradiation or hyperthermia alone was not detrimental for anastomotic healing (6). These findings were supported by the microscopic findings in this study.
Anastomotic healing after colonic resection and anastomotic reconstruction can be regarded as a normal wound repair process. The operative trauma causes an inflammatory reaction, with fibrin clot formation, edema, polymorphonuclear infiltration, macrophage reaction and collagen formation by fibroblasts (11). Collagen, mainly present in the submucosal layer, provides mechanical strength to the anastomosis. The collagen content (measured by the hydroxyproline content) is generally regarded to be a good indicator for quantification of anastomotic healing (11,12).
During the process of experimental colonic wound healing mechanical strength has returned to normal values within seven days after surgery. This can be observed in experimental anastomoses which are tested for bursting strength; after one week the colon usually ruptures outside the anastomosis, indicating that the normal (peri-anastomotic) tissue can be considered weaker than the anastomosis (6,12). Disturbances of this normal wound

healing can be expected in the first few days, or by lengthening of the normal healing time.
In this study, the effects of treatment with irradiation were clearly visible in the distal limb of the anastomosis. Treatment with irradiation or heat alone was not detrimental for early anastomotic strength (6), and only moderate histological disturbances were apparent. The histological changes in normal tissues after single modality adjuvant treatment are not the same: irradiation alone leads to marked submucosal edema, mucosal necrosis, a polymorphonuclear leucocyte reaction and (allthough not investigated here) micro-angiopathic changes of the bowel, which is considered to be in part responsible for late radiation side effects, whereas hyperthermia appears to have only a limited effect, with little mucosal edema and local inflammatory reaction.
In the I/H group the combination of both preoperative irradiation and hyperthermia led to increased wound healing problems in the anastomotic area in the form of increased necrosis formation, massive infiltration and insufficient anastomotic healing (leakage) in some of the animals. Earlier observations of macroscopical transmural necrosis and wound healing problems after combined irradiation and hyperthermia (6) were therefore supported in this study. However, it was remarkable that in the I/H group next to disturbed anastomotic healing in some rats, there was also relatively normal healing present in others.
This indicates that the chosen treatment, i.e. 25 Gy irradiation followed by 30 min at 44°C, is at the treshold between normal and insufficient repair. Healing may go either way. Variations in vascular supply may be responsible for this effect. It should be kept in mind that the time interval between irradiation and heat is very important as well (2).
Despite the relatively small number of animals in each group, some marked differences could be observed between groups sacrificed after 3 or 7 days. The inflammatory reaction with polymorphonuclear leucocytes subsided after 7 days in the I and C groups, and the anastomotic area showed a less disturbed aspect. This was not true for animals in the H and I/H group.
It is remarkable that the count of collagen fibrils in the anastomotic area was equal in all groups, whereas in a former study the chemical measurement of the hydroxyproline content (representing collagen) in the I/H group was significantly higher than in the control group (6). In the 3 days group a

tendency to this extent is seen, with the small group preventing reliable statistical evaluation. This difference may be explained by the fact that visual collagen measurement is a method using only one part (one slice) of the entire circumference of the anastomosis examined, and there is no correction for circular differences. On the other hand, in fibril counts the collagen outside the anastomosis is not measured, leading to more accurate figures than the chemical assay of the entire anastomosic segment which also contains uninjured intestine.

The treatment method used in our experiments, using intracavitary hot water for the hyperthermia treatment, has not been studied before, and the pathophysiology of the injury to the colonic tissue following combined irradiation and this heat application is still not completely understood.

Normal tissue sensitivity for radiation beams is not equal in different tissues, and this determines the maximum dose of radiation that can be used. It should be emphasized that the same principle is true for heat sensitivity: in order to prevent serious side effects the total amount of heat used (both in time, temperature, fractionating of the heat application etc) should be different according to the type of animal or tissue and the fact whether irradiation is or will be used. Studies using other tissues or other treatment characteristics can not be simply translated to other models.

In the small bowel in rats it has been shown that heat alone, applied with water of 44°C for 30 min, leads to a granulocyte infiltration until three day post-heating, and fibrosis occurring from the seventh day onwards (7). In a microscopical study in swine, 30 min of intracavitary microwave heat application led to injury that was correlated with temperature. First (43°-44°C) only focal, superficial necrosis of the mucosa was present, which was considered repairable. When the intracavitary temperature was higher (45°-46°C), edema was abundantly present, together with congestion and increasing amounts of mucosal necrosis. The use of higher temperatures was complicated by transmural necrosis (mucosal burn wound) (13). These data may not be comparable to our study since in our model we used intracavitary hot water which may lead to a different degree of heat penetration and heat absorption; also the rat bowel wall is much thinner than the bowel wall of swine.

In oncological studies heat and radiation therapy have proven to act pathophysiologically in different ways (14). Heat has a direct killing effect

on all cells, with the ultimate effect a third degree burn wound. The rationale for using heat is that tumors have parts which are less vascularised and harbour hypoxic, radioresistant cells; cooling by the bloodstream is not efficient, resulting in a higher local temperature and higher probability of cell kill. Well-vascularised tumor parts are radiosensitive because they are well-oxygenated and have a microenvironment with normal pH, leading to increased cell-kill by irradiation. As a result these mechanisms lead to an additive effect when both modalities are used with short intervals

This study has demonstrated the outcome after borderline adjuvant therapy - both the single radiation dose of 25 Gy and heat treatment with water of 44°C for 30 min are at the upper limit of tolerance in rats. A dose-effect study, with emphasis on the time interval between irradiation and hyperthermia, and investigation of late effects, is now under consideration

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Chapter 6

EFFECT OF IRRADIATION ON HEALING OF NEWLY MADE COLONIC ANASTOMOSES IN THE RAT

J.Biert[*], Th.Wobbes[*], Th.Hendriks[*], J.Hoogenhout[#]

From the Departments of [*]Surgery and [#]Radiotherapy, University Hospital Nijmegen, the Netherlands

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ABSTRACT

Purpose short-term effects of radiotherapy on the healing process of newly made colonic anastomoses are investigated by measuring the anastomotic strength in a rat model.
Methods and materials: four groups of Wistar rats were used. In all groups, rats underwent a 1 cm sigmoid resection with end-to-end anastomosis. Group I served as a control group. In group II the anastomosis was irradiated after closure of the abdominal wall with a single dose of 20 Gy of 250 kV X rays. Group III was irradiated with a single dose of 20 Gy while the abdominal wall was not closed, and the surrounding tissues were carefully covered by a lead plate, simulating intra-operative radiotherapy. Group IV was treated as group III, but a larger dose of 25 Gy was applied. Animals were sacrificed 3 or 7 days after the operation. General condition of the rats was determined by observation, weight loss, serum protein and albumin at sacrifice. Anastomotic healing was evaluated by inspection, bursting pressure, hydroxyproline and protein contents of the anastomotic segment.
Results: direct post-operative externally irradiated rats (group II) showed a marked weight loss, hypoproteinaemia and hypo-albuminaemia because of involvement of small bowel in the irradiated volume. With respect to anastomotic healing there were no significant differences between control and irradiated groups.
Conclusions: these data suggest that the application of a single dose of irradiation (20 and 25 Gy) on colonic anastomoses given in a direct postoperative or intra-operative model has no measurable side effect on the early healing of newly made colonic anastomoses. Direct post-operative external irradiation results in unwanted side effects in the adjacent bowel.

INTRODUCTION

A combination of surgery and intra-operative radiation therapy (IORT), high dose intra-operative brachytherapy, or direct postoperative external radiotherapy is subject of increasing interest in both experimental studies and clinical trials (1-12). It is expected that this mode of treatment will be used more extensively in the future because of theoretical and practical advantages (6). Several clinical studies have been started investigating the efficacy of intra-operative radiotherapy in the treatment of intra-abdominal or other malignancies (1,2,6). A high single dose is given on the exact location of the tumor bed after surgical dissection; other tissues, like the small bowel, can be carefully protected from unwanted side effects of irradiation. Combined with fractionated external irradiation the total effective radiation dose is increased as compared to fractionated doses used over several weeks after operation only. Moreover, direct irradiation has the advantage that residual tumor cells will be attacked immediately, yielding

in a greater probability of local control. There have been some reports of intra-operative irradiation in colorectal surgery (1-3,12). If a low anterior resection is performed, lateral and distal cutting edges are very important for obtaining local control of disease. Use of IORT could be very helpful in situations where local recurrence is more likely to appear, as in resection of big tumors, and peroperative tumor cell spill. Since the remaining rectum can not be held aside, irradiation of anastomotic tissue is unavoidable. The question arises if under these circumstances anastomotic repair can be done safely.

A number of animal studies have confirmed the deleterious effects of preoperative irradiation on healing of large bowel anastomoses (13-16). Although radiation dose and regimen, and time between irradiation and surgery vary widely in the various studies reported, the general picture that emerges is that preoperative irradiation almost invariably appears to impair anastomotic healing as assessed by incidence of leakage or measurement of anastomotic strength.

Unlike the long-term effects on several tissues little is known about the direct or short-term effects of irradiation on the healing of newly made intestinal anastomoses in animal models. In our view experimental studies of colon anastomotic wound healing while radiotherapy is applied are mandatory to provide more insight in healing processes under these circumstances. For this purpose we used a rat model to examine the healing of newly made colonic anastomoses after segmental colon resection and the application of IORT or direct post-operative radiotherapy.

METHODS AND MATERIALS

Experimental animals

Eighty male Wistar rats (250 grams ± 5 %) of the outbred strain C_{pb}:WU were divided into four groups of 20 animals each. They were provided with a standard diet (Hope Farms, Woerden, the Netherlands) and allowed water ad libitum.

Operative procedure

While under anaesthesia (sodium pentobarbital intra-peritoneally) animals

were held in a special frame which allowed a standard way of presentation of all anatomical structures. A median laparotomy was performed and 1 cm of colon was resected 2 cm proximal from the rectalperitoneal reflection. Continuity was restored by an inverting one-layer end to end anastomosis with 8 interrupted monofilament sutures (Ethilon 8-0, Ethicon®, Norderstedt, Germany) using microsurgical techniques. In groups I (control group) and II fascia and skin were closed immediately; in groups III and IV this procedure was performed after IORT.

Irradiation techniques and dosimetry

Accurate localisation of the anastomoses in group II was done in a seperate group of ten rats. By means of radiographs it was shown that the anastomoses, marked with radio-opaque clips, were always located within a small field of 2.2 cm^2 (Figure VI-1). To cover the rest of the body a lead shield (thickness 2,5 mm) was used with a window of the same size. Figure VI-2 illustrates the peroperative irradiation set-up of the animals.

Radiation dosimetry was performed by means of thermoluminescent dosimeters and film densitometry in separate animals. Irradiation was performed with a 250 kV X ray unit with a 1 mm Cu filter (target-colon distance 25 cm). The dose rate was 1,19 Gy/min in group II and 1,29 Gy/min in groups III and IV.

Treatment schedules

In all animals a segmental colon resection was performed. After this, animals in group I were sham-irradiated; they served as a control group. In group II animals were irradiated with a single dose of 20 Gy after closure of fascia and skin. Animals in group III and IV were irradiated intra-operatively, that is, before closure of fascia and skin. Here the anastomosis was held apart in a special lead cone in order to prevent unwanted irradiation of adjacent intestine, which was carefully removed from the irradiation field. Single doses in groups III and IV were 20 Gy and 25 Gy, respectively. During irradiation all animals were held under anaesthesia to prevent movement of the target area. Animals were sacrificed on day 3 or day 7 by cardiac puncture (n=10 for every group).

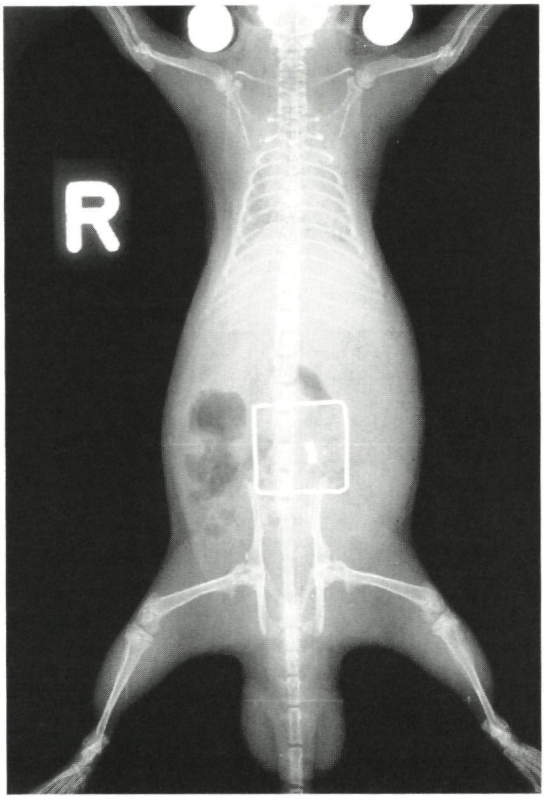

Figure VI-1. Group II, pilot study. Röntgenogram showing a radioopaque clip marking the anastomosis, and a frame which depicts the outline of the irradiation field. Localisation is shown to be correct.

Assessment of results

The condition of the animals was monitored by weighing every day, and blood samples were taken at sacrifice for determination of serum protein and albumin levels. The abdomen was inspected for abnormalities. The anastomoses were resected en bloc. Anastomotic bursting pressure was measured. The segment of colon containing the anastomosis was connected to an infusion pump which contained methylene blue stained water. Using a standard infusion rate of 2 ml/min the colon segment was slowly inflated until leakage occurred, which caused the pressure to fall. To visualise the precise bursting site the colon segment was held under water. The highest pressure obtained was called the bursting pressure. Anastomotic healing was assessed by measurement of anastomotic strength and hydroxyproline content (17); the latter is a measure for the amount of collagen present.

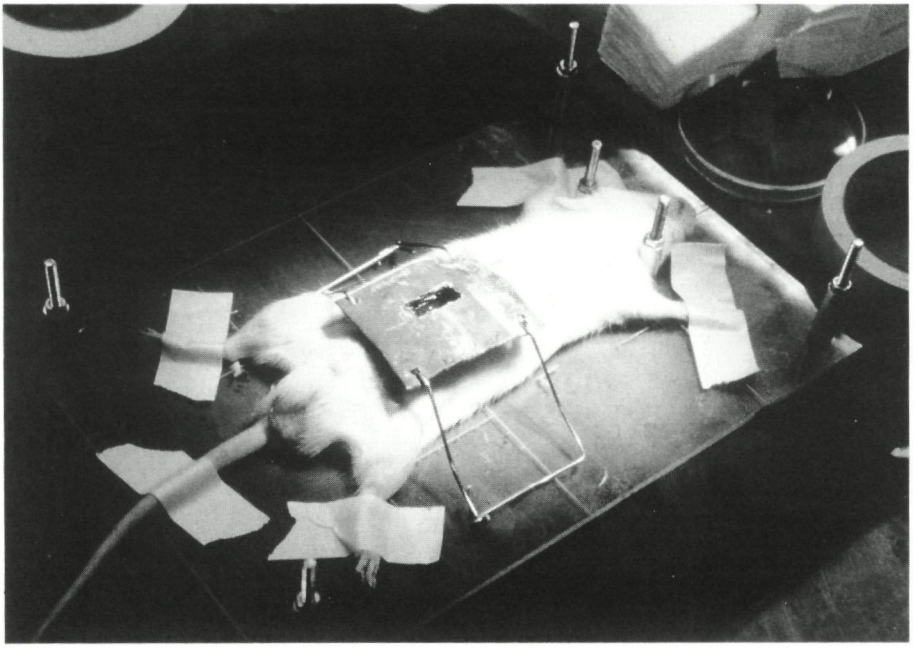

Figure VI-2. Experimental set-up showing immobilization of the animal, and the lead shield cone covering the surrounding tissues.

A 1 cm segment of colon containing the anastomosis was cleaned from adhesions and stored in liquid nitrogen. Subsequently, these tissue samples were weighed, pulverised, lyophilised and kept at -30° C until analysis. In each rat both the control segments removed at operation and the samples containing the anastomosis were analyzed for hydroxyproline as described before (18) essentially according to the method described by Prockop and Udenfriend (19). Protein levels were assayed according to Lowry *et al.* (20), using bovine serum albumin as a standard. For statistical analysis Wilcoxon and Kruskal-Wallis tests were used.

RESULTS

General observations

In group II one rat died of unknown cause during the experiment after 4 days. In the irradiated groups all rats had moderate diarrhoea.

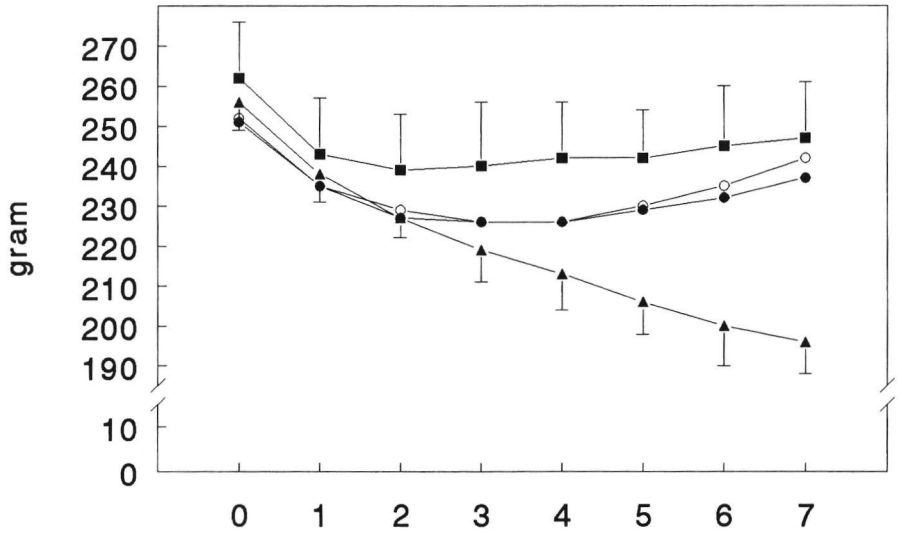

Figure VI-3. Change in body weight during the experiment. Open circels: control group (I). Triangels: 20 Gy direct postoperatively (II). Closed circels: 20 Gy IORT (III). Squares: 25 Gy IORT (IV). SD shown in groups II and IV.

Body weight

Post-operative changes in body weight are depicted in Figure VI-3. There were no significant differences between groups at the start of the experiment. Up to 3 days, most animals lost some weight (up to 10 % of body weight before operation). Thereafter, rats in groups I, III, and IV started gaining weight again. After 7 days the weight of these rats was still under the level at operation, but it was rising steadily. Only the animals in group II lost progressively more weight over the entire experimental period ($P < 0{,}005$ as compared to all other groups on day 7, Wilcoxon test).

Serum albumin and protein

Average levels for serum albumin and protein are given in Table VI-1. A comparison of the four experimental groups, together with a group of non-operated controls of equal body weight (normal values), showed significant differences (Wilcoxon test). In general, average values were always lower in the operated animals than in the non-operated controls.

In particular, the protracted weight loss exhibited by group II animals was associated with an even stronger decrease in levels of serum albumin and protein

Two-by-two comparison of groups showed the levels in group II to be significantly (P<0,001 compared to groups I, III, IV and to non-operated controls; Wilcoxon test) lower than in any of the other groups at 7 days after operation.

Table VI-1. *Serum protein and albumin levels (average ± SD) at day of sacrifice*

	serum protein *(g/l)*		serum albumin *(g/l)*	
	day 3	day 7	day 3	day 7
normal values	62 6 ± 2 4	62 6 ± 2 4	31 4 ± 0 8	31 4 ± 0 8
group I	56 3 ± 1 3	55 9 ± 2 5	26 5 ± 0 8	26 9 ± 1 4
group II	49 5 ± 3 3*	39 6 ± 3 5*	23 0 ± 1 5*	16 6 ± 1 6*
group III	52 3 ± 3 3	52 0 ± 3 2	23 8 ± 1 8	24 0 ± 2 3
group IV	53 5 ± 2 6	54 8 ± 4 7	26 6 ± 1 3	26 6 ± 1 9

Normal values are taken from non-operated weight-matched control rats

* P<0 001 (Kruskal-Wallis and Wilcoxon tests) as compared to all other groups

Anastomotic strength

Results of bursting pressure measurements (mm Hg) are depicted in Figure VI-4.

In group I the measurement of the bursting pressure after 3 days always resulted in anastomotic rupture with low values. The average bursting pressure was 108 ± 42 mm Hg. In group II bursting pressures averaged 107 ± 35 mm Hg (9/9 ruptures inside the anastomosis), and in group III 98 ± 43 mm Hg (9/10 ruptures inside the anastomosis). In group IV the average bursting pressure was 128 ± 45 mm Hg, but here only 6/10 ruptures were inside the anastomosis. Thus, in measuring the bursting pressure at 3 days there were no significant differences between the four groups (Kruskal-Wallis test). One rat in group II was excluded because of technical failure while performing the measurement.

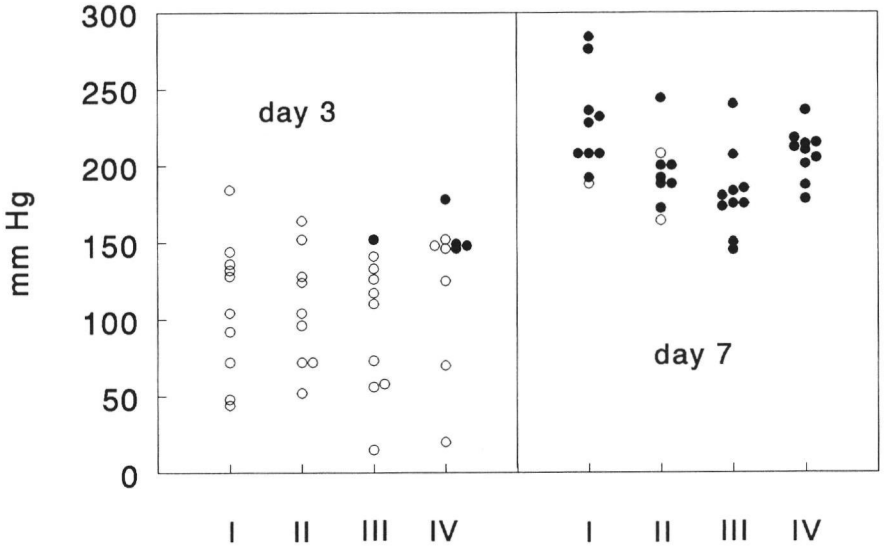

Figure VI-4. Bursting pressures of anastomotic segments. Open circels: bursting site within the suture line. Closed circels: bursting site outside the suture line. I: control group. II: 20 Gy direct postoperatively. III: 20 Gy IORT. IV: 25 Gy IORT.

On day 7 almost all bowel segments ruptured outside the anastomosis, indicating that the anastomotic strength was higher than the strength of the adjacent colon.

Table VI-2. *Protein content of control and anastomotic segments*

	day 3		day 7	
	control	anastomosis	control	anastomosis
group I:	11.9 ± 1.7	27.7 ± 7.1	12.8 ± 1.7	32.7 ± 8.0
group II:	11.6 ± 1.0	21.3 ± 4.3	12.5 ± 0.7	23.6 ± 3.7*
group III:	10.4 ± 1.1	25.6 ± 5.1	10.9 ± 1.4	32.7 ± 5.1
group IV:	12.0 ± 1.4	20.1 ± 4.0	12.4 ± 1.3	30.7 ± 7.6

Results are expressed as average value in mg per cm tissue ± SD
*: P=0.01 (Kruskal-Wallis and Wilcoxon tests) as compared to all other groups

Protein - hydroxyproline

In all bowel segments of 1 cm which were removed at the operation and those segments which contained the anastomoses protein content (Table VI-2) and hydroxyproline content (Table VI-3) were compared. There were no significant differences between the contents of the control segments (Kruskal-Wallis test).

Table VI-3. *Hydroxyproline content of control and anastomotic segments*

	day 3		day 7	
	control	anastomosis	control	anastomosis
group I	199 ± 18	334 ± 65	209 ± 25	530 ± 124
group II	212 ± 40	331 ± 51	203 ± 19	449 ± 55
group III	167 ± 17	326 ± 34	168 ± 31	531 ± 88
group IV	192 ± 22	312 ± 39	210 ± 23	603 ± 116

Results are expressed as average value in μg per cm tissue ± SD

In all groups there was an increase in protein and hydroxyproline content between days 3 and 7 in the segments containing the anastomosis. Only group II showed a significantly lower value of protein content on day 7 (P=0,01) as compared to the other groups (Wilcoxon test). However, this was not the case for measuring the hydroxyproline content although the mean value was lower than that of the other groups.

In group IV the increase in protein and hydroxyproline content of the anastomosic segment was higher than in the other groups, but there was no significant difference with the control group (Wilcoxon test).

DISCUSSION

It is well-known that irradiation of the abdomen can have serious consequences. There are early side effects, like enteritis of the small and large bowel on basis of mucosal damage, and reduced strength of intestinal anastomoses and skin wounds, if constructed afterwards (21-25). Late

effects on basis of late radiation enteritis comprise formation of fistula's and fibrotic and stenotic changes of the gastrointestinal tract (4,26).
There are also effects on surrounding or underlying organs, which cannot be shielded. This phenomenon in particular limits the application of high single dose external irradiation. In case of intra-operative radiotherapy the surrounding tissues can be kept outside the field of irradiation, thus allowing the deliverance of a relatively high dose of irradiation (10-25 Gy) to the tumor bed after resection. Little is known about the direct effect of a high single dose of irradiation on intestinal anastomoses; experience is limited to animal studies, since there are no specific reports about anastomotic healing when IORT is applied. Animal studies concentrate on the question how to find a balance between increasing radiation doses and maintaining acceptable morbidity and mortality (13).
In most animal studies on wound healing preoperative irradiation was applied (14-16). Fewer studies report about experiments with intra-operative irradiation directly after resection. Sindelar and his group investigated anastomotic healing in dogs (11,27,28). In their study there was a deteriorating effect on intestinal wound healing above a single dose of 30 Gy. Irradiated blind jejunal loops were used. Poulakos studied intra-operative irradiation of the duodenum in rats (7,8), and found that single doses of 20 Gy or more were associated with unacceptable high incidence of late complications.
The aim of that study was the effect of irradiation of the pancreatic region for carcinoma with possible side effects on the duodenum. Studies were mostly histological, and no anastomoses were made. Recently, Saclarides reported impaired healing of small bowel anastomoses in rats after a single dose of 20 Gy given intra-operatively (9,10). In this case, one or both limbs were irradiated before construction of the anastomosis. Fibrin glue, applied on the anastomosis, improved healing.
In this study we focused on the effect of irradiation on healing of newly-made colonic anastomoses. Single irradiation doses of 20 and 25 Gy were applied. This treatment schedule was chosen because we wanted to investigate the effects of irradiation in a dose range where normal healing of the anastomosis is questionable. When looking at the human situation the tolerance dose (TD 5% severe complications in 5 years) for acute effects of human intestine is approximately 50 Gy in daily fractions of 2 Gy.

According the linear quadratic formula and assessing an α/β ratio of 10 Gy for acute effects (29,30), the extrapolated dose of 60 Gy is about the biological equivalent for acute effects with a single dose of 20 Gy. A single dose of 25 Gy is above the accepted tolerance dose for human intestine. The doses of 20 and 25 Gy used in the present experiment were chosen to investigate the borders of acceptable irradiation dose, when compared to the human situation.

It does not seem realistic to increase the dose of irradiation because earlier investigations showed detrimental late effects on intestinal mucosa in a later stage after such high doses (8,31). Earlier experiments in our clinic concerning anastomotic wound healing under different conditions have shown that faecal leakage, bursting pressure, hydroxyproline and protein content of the anastomosis are parameters of wound healing, which allow us to assess anastomotic healing in this experiment (17). It is always hard to prove that animal models are representative for the human situation. Other investigators have found rat models useful for irradiation research because the intestinal tissue responded correlating to dosage, irradiation dose rate, and microscopic damage, like in preoperative irradiation (7-9,15,16). This is why we consider our rat model to be useful.

In group II which received 20 Gy directly after closure of the skin there were indications that a relatively large part of the surrounding intestine was involved; this resulted in a very low serum protein and albumin concentration and a low body weight after 1 week which was equal to the effect of protein starvation for more than 7 weeks (32). Despite this situation we found no decrease in bursting pressure or difference in protein and hydroxyproline content. In group III and IV we irradiated the anastomosis directly while the abdominal cavity was still open and all other intestinal tissue could be protected. In these groups there was no sign of malnutrition. Healing after 20 or 25 Gy showed no significant differences in bursting pressure. On the contrary there was a tendency after 25 Gy to burst outside the anastomosis after 3 days, indicating that the strength of the anastomosis was higher than the bursting pressure of the adjacent colon because of a strong fibrotic reaction. After 7 days there were no large differences between control and irradiated groups (Figure VI-4).

The data in this experiment indicate that early healing of rat colonic anastomoses is not impaired after single doses of 20 or 25 Gy, neither

directly applied on the anastomosis, nor after closure of the abdominal wall. Anastomotic strength, measured by bursting pressure and collagen production, is not impaired. Expectations about a negative effect of irradiation in a direct postoperative model were not confirmed by this study. The basic pattern of wound healing is similar in every tissue; it can be divided into three stages: a lag phase, a proliferation phase and a maturation phase. In undisturbed wounds the influx of macrophages in the wound area is observed 24 hr after wounding. These cells regulate degradation and synthesis of collagen, which processes eventually result in the restoration of wound strength (33). In our experiments the radiation dose was given immediately after wounding, that is, before unirradiated macrophages would enter the wound area. Because of a time interval between irradiation and this influx we think that wound healing may not be influenced by the radiation dose. This explains the absence of the previously expected negative impact of peri-operative irradiation.
The question of possible late side effects of IORT is not examined in this experiment; since this is also important for the safety evaluation in the use of IORT, it should be subject of further investigation.

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Chapter 7

LATE EFFECTS OF INTRA-OPERATIVE RADIATION THERAPY IN ANASTOMOTIC RAT COLON

W.F.Seifert[*], J.Biert[*], Th.Wobbes[*], A.A.J.Verhofstad[§], J.Hoogenhout[#], Th.Hendriks[*]

From the Departments of [*]Surgery, [§]Pathology and [#]Radiotherapy, University Hospital Nijmegen, the Netherlands

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submitted

ABSTRACT

Purpose to determine whether intra-operative radiotherapy causes long-term side effects on healing of colonic anastomoses in the rat
Material and Methods 175 rats were divided into seven groups One group (C) served as sham-irradiated control group, all others were irradiated Following a colonic resection, 1 or 2 cm of the distal bowel end was irradiated with a single radiation dose of 10, 15 or 20 Gy (groups 10/1, 15/1, 20/1, 10/2, 15/2 and 20/2 respectively) Subsequently, an anastomosis was constructed The animals were killed after 6 (n=10 in each group) or 12 months (n=15 in each group) The abdomen was inspected for abnormalities and the colonic diameter was measured A 1 5 cm colonic segment containing the anastomosis was removed and divided longitudinally in 2 equal segments One part was used for hydroxyproline determination, while the other part was used for histological analysis
Results During the experimental period, 1 rat (group 15/1) died because of anastomotic leakage, 3 others died from unknown causes There was no difference in colonic diameter between groups After 6 or 12 months, 17 rats had developed an adenocarcinoma in the irradiated area, 11 of these had received a radiation dose of 20 Gy Microscopic observation indicated that fibrosis was only present in a limited number of animals, especially after irradiation with a dose of 15 or 20 Gy The anastomoses had healed normally The hydroxyproline content of the anastomotic segment was higher in the 20/2 group after 6 months as compared to the control group In the distal anastomotic segment (the irradiated part) the hydroxyproline concentration was higher in the 10/1 and 15/1 groups after 12 months as compared to the control group Otherwise there were no differences between groups
Conclusion Intra-operative irradiation of one limb evoked dose-related changes (formation of adenocarcinomas and fibrosis) and time- or volume-related changes (adenocarcinomas) in anastomotic segments Bowel stenosis or other late side effects did not occur Anastomotic wound healing was uneventful

INTRODUCTION

Intra-operative radiation therapy (IORT) may be a useful innovation of conventional radiation therapy in patients with large bowel cancer (1-3). A moderate to large dose of radiation is provided to the tumor bed directly after surgical resection of the primary tumor. The advantage of this approach over conventional fractionated external radiation therapy is the limited irradiated volume while the risk of radiation damage of surrounding healthy tissues is reduced by shielding and surgical mobilization. However, the combination of colorectal surgery and IORT is associated with increased risk of complications to the intestine since the remaining distal rectum

should not be shielded and has to be incorporated into an anastomosis. In a previous study, we have investigated the effects of this therapy on early anastomotic repair since suppressed healing could increase the risk for anastomotic dehiscence, which is a serious complication with a concomitant high morbidity and mortality rate (4,5). A radiation dose of 25 Gy delayed the development of early wound strength in experimental colonic anastomoses if the bowel ends were irradiated intra-operatively before anastomotic construction (6). Interestingly, this was not the case if the anastomosis was irradiated immediately after construction (7). In these studies, we examined radiation side effects only during the first two postoperative weeks. To date, there are no experimental data available on potential late side effects on colonic anastomoses resulting from intra-operative irradiation. Late intestinal complications from conventional fractionated external radiation therapy have been recognized but have not been studied extensively. Clinically it may take years to develop these side effects but a median of about 12 months has been reported before the injury becomes apparent (8). Frequent and urgent stools, blood loss and abdominal cramping are common symptoms. Microscopically most changes are noted in the submucosa of the intestine (8,9). Focal areas of stenosis, ulcerations and increased deposition of collagen (fibrosis) have been observed in areas of the irradiated bowel. Atypical vascular changes such as thickening of vessel walls have also been noticed.

The impact of time, radiation dose and volume on the risk of large bowel side effects has been estimated before in several animal studies (10-13). However, in these studies no anastomoses were made. The aim of the present study was to evaluate - clinically, histologically and biochemically - late effects of IORT in anastomotic rat colon. Clinically, IORT has been applied using irradiation doses in the range between 10 and 40 Gy (1-3). Since we already established that a dose of 25 Gy is detrimental to early anastomotic repair (6), doses of 10, 15 and 20 Gy were chosen. In addition to the dose, the irradiated volume was also varied.

METHODS AND MATERIALS

Animals

Three-months-old male outbred Wistar/Cpb:WU rats, weight 277 ± 18 gram (mean ± SD, n= 175), were obtained from our own colony (Nijmegen, The Netherlands). The animals were housed in groups of two in Makrolon type 3 cages. Water and a standard laboratory food (Diet AM II, Hope Farms, Woerden, The Netherlands) were supplied ad libitum. The body weight was recorded daily during the first two weeks after operation and once a month thereafter. All signs of illness, reaction to treatment and mortality were recorded. The study was approved by the Animal Ethical Review Committee of the Faculty of Medical Sciences, University of Nijmegen.
After a seven-day pre-experimental period the rats were randomly divided into seven groups of 25 rats: a control group that underwent a sham-irradiation procedure before anastomotic construction (C) and six groups where 1 or 2 cm, respectively, of the distal margin of the bowel was irradiated with a dose of 20, 15 or 10 Gy, respectively, before anastomotic construction (Figure VII-1).

Operative procedure

At the day of operation the rats were anaesthetized by an intra-peritoneal injection of sodium pentobarbital (50 mg/kg). Surgery was performed under semi-sterile conditions using a Zeiss operation microscope. The abdominal skin was shaved, disinfected with 70% ethanol, and a median laparotomy of 4 cm was performed. In each animal 1 cm colon was resected at 3 cm proximal to the recto-peritoneal reflection. Intra-operative irradiation was performed on the distal segment held apart in a lead cone to prevent unwanted irradiation of adjacent tissue as described before (6). A volume of 1 or 2 cm in length of the distal segment was irradiated (Figure VII-1). A dose of 10, 15 or 20 Gy was delivered by a 250 kV X-ray unit with a 1 mm Cu filter at a dose rate of 1.29 Gy/min. Thus, six experimental groups were formed (groups 10/1, 15/1, 20/1, 10/2, 15/2, 20/2). The control group underwent sham-irradiation before anastomotic construction. An end-to-end anastomosis was constructed using 8 single layer inverting interrupted 8-0 Ethilon (Ethicon®, Norderstedt, Germany) sutures. The abdomen was closed using a 3-0 silk suture for the fascia and staples for the skin.

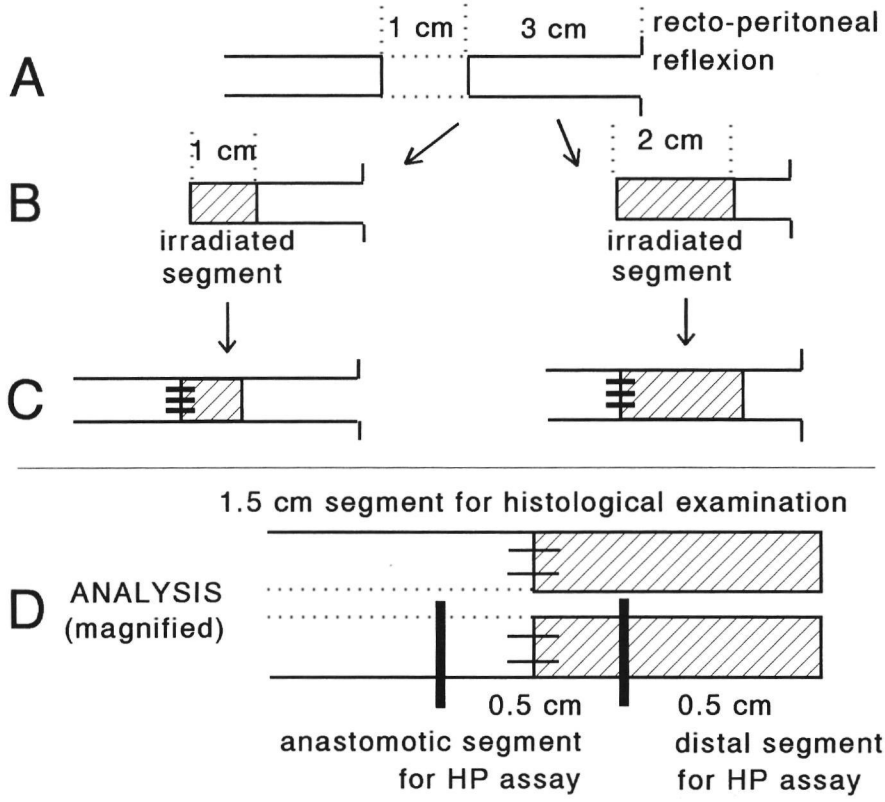

Figure VII-1. Schematic representation of experimental procedure. **A**. Laparotomy plus resection of 1 cm colon. **B**. Irradiation (dosis 10, 15 or 20 Gy) of a 1 or 2 cm distal segment. **C**. Construction of anastomosis with only the distal segment consisting of irradiated tissue. **D**. At sacrifice: samples used for histological and hydroxyproline (HP) analysis.

Ten animals of each group were killed at 6 months and 15 at 12 months after surgery. Macroscopic examination of the irradiated colon and anastomoses was performed in situ. Thereafter, the colon including the anastomoses was isolated. A 1.5 cm colon segment, including the anastomosis in the centre, was divided longitudinally into two equal parts (Figure VII-1). One of the samples was pinned on a plastic grid and immersed in 4% phosphate buffered formalin (pH 7.4) for histological examination. The other sample was subdivided into three parts of 5 mm each: one segment including the anastomosis, a non-irradiated proximal part and an irradiated distal piece. All parts were weighted and frozen in liquid nitrogen for determination of the hydroxyproline content.

Histological examination
The tissue was routinely processed and embedded in paraplast. The sections with a thickness of 4 μm were stained with Hematoxylin-Eosin, or Sirius Red F3BA for collagen (14), and were scored for the presence of histological changes by light microscopy. The parameters examined were the presence of cell necrosis, inflammation, structural changes of crypts, mucosal ulcerations, fistula, stenosis of the colon, the presence of increased collagen in the anastomotic area (fibrosis), the thickening of the blood vessel walls and the presence of malignancies.

Anastomotic collagen
Anastomotic samples and their proximal and distal parts were lyophilized, weighted and pulverized. The hydroxyproline content and concentration, as a measure for collagen, was measured by HPLC after hydrolysis with 6N HCl and derivatisation with dabsylchloride.

RESULTS

General observations
All animals survived the operative procedure. Independent of the radiation dose and the irradiated volume, a mean weight loss of about 9% was noticed at the first postoperative day (not shown). Thereafter, the average body weight of all rats gradually increased without any significant difference between control and experimental groups. At 6 and 12 months after operation the rats had gained weight from 277 ± 18 to 451 ± 14 and 541 ± 8 g, respectively.
Clinical symptoms of early radiation injury of the colon were observed in 7 rats: 2 rats in group 15/1, 1 in group 20/1, 2 in group 10/2, and 2 in group 20/2 suffered from mild diarrhoea during the first postoperative week. Three of these rats had some bloody discharge. All rats had recovered in the second postoperative week. During the experimental period 2 rats in group 15/1, 1 in group 20/1, and 1 in group 20/2 died spontaneously; 1 of these rats (group 15/1) died because of the complications resulting from anastomotic leakage at 12 days after surgery. The other 3 died 2, 9 and 11 months after operation (groups 15/1, 20/1 and 20/2, respectively); however,

this was not related to late gastrointestinal complications of radiation therapy.
Macroscopic examination of the anastomoses at the day of killing revealed some signs of late radiation injury. Mild to moderate ileal-colonic adhesions (peri-anastomotic fibrosis) were observed in 7 irradiated rats at 6 months and in 5 irradiated rats at 12 months (8% of the total number of irradiated rats) at the site of the irradiation.
The in-situ quantification of the proximal, anastomotic and distal diameter of the colon revealed no differences between control and irradiated groups (Table VII-1). The diameter of the colon distal from the anastomosis was always smaller than that proximal to the anastomosis. This effect was also observed in the control group.

Histology
Histological examination of the anastomoses at 6 and 12 months after operation revealed no differences for most of the parameters between the control and irradiated groups. In the anastomotic areas, ulceration did not occur, neither were fistula, stenoses or perforations observed. The normal pattern of the mucosal layer and lamina propria was present. Structural changes of the muscle layers and submucosa were observed in the anastomotic area. However, they were not related to the radiation treatment, since the same was seen in the control animals. In all animals, the disconnection at the site of the anastomosis in the muscularis mucosa and propria remained present. Although muscle cells were observed between both limbs, many fibroblasts in scar tissue had filled the anastomotic area. At the site of the submucosa highly differentiated scar tissue was found in all groups.
Table VII-2 shows histological parameters observed in control and irradiated colon. Fibrosis, measured as the presence of Sirius Red stained collagen fibrils, was found in a minority of animals within each group. Still, its presence appeared to be dose-dependent since it occurred in only 1/25 animals in the sham-irradiated control group, and in 2/50, 7/48 and 10/48 animals after irradiation with a dose of 10, 15, or 20 Gy, respectively. Also, some of the larger arteries in the irradiated area showed signs of intimal fibrosis. Furthermore, adenocarcinomas were present in the irradiated submucosa in 17/146 of the irradiated animals.

The presence of adenocarcinomas in the anastomotic area was related to the irradiated volume, the radiation dose, and the time after irradiation. They were observed in 11 rats after irradiation of 2 cm bowel, against 6 rats after irradiation of 1 cm bowel. Irradiation with a dose of 0, 10, 15, and 20 Gy led to adenocarcinomas in 0, 2, 4 and 11 rats, respectively. Also, 6 tumors were present after 6 months, and 11 after 12 months. There was one double tumor (Figure VII-2).

Signs of inflammation were hardly observed at all, although severe inflammation was present in 4 rats after a radiation dose of 15 or 20 Gy on 2 cm colon.

Table VII-1. *Diameter (mean ± SD) of proximal colon, anastomosis, and distal colon at 6 months and 12 months after surgery*

	mean diameter (mm ± SD)		
group	proximal	anastomosis	distal
6 months			
C	6 2 ± 2 0	6 7 ± 1 8	5 7 ± 1 7
10\1	5 9 ± 2 4	6 3 ± 2 0	5 8 ± 2 0
15\1	5 9 ± 1 1	6 0 ± 1 6	5 4 ± 1 6
20\1	6 5 ± 2 2	6 7 ± 1 5	5 0 ± 1 4
10\2	5 6 ± 1 8	6 1 ± 1 6	5 0 ± 1 5
15\2	6 3 ± 2 3	7 0 ± 1 9	5 5 ± 1 3
20\2	6 1 ± 2 1	6 5 ± 1 1	5 2 ± 1 5
12 months			
C	5 8 ± 1 4	6 4 ± 1 4	5 4 ± 1 4
10\1	5 5 ± 1 4	6 3 ± 1 0	4 7 ± 0 9
15\1	5 6 ± 1 0	6 4 ± 2 0	4 9 ± 1 1
20\1	6 1 ± 1 6	6 4 ± 1 7	5 1 ± 1 5
10\1	5 7 ± 1 3	6 5 ± 1 1	5 3 ± 1 2
15\2	5 7 ± 0 9	6 2 ± 1 0	4 9 ± 1 0
20\2	6 4 ± 2 1	7 2 ± 2 0	5 2 ± 1 1

Anastomotic collagen

The segments containing the anastomosis, and the distal, irradiated segments were assayed for hydroxyproline concentration and content as a measure for collagen. The median values for both hydroxyproline concentration and content in anastomotic and distal segments, are given together with the 5-95% and 25-75% interval in Figures VII-3 and VII-4, respectively. In the anastomotic segments of all groups, both concentration and content were always significantly higher at 12 months than at 6 months after operation ($P<0.05$; two-tailed Mann-Whitney U test, Figure VII-3).

Table VII-2. *Histological examination of colonic anastomoses at 6 months and 12 months after surgery*

group	n	adenocarcinoma	fibrosis	inflammation
6 months				
C	10	0	0	0
10\1	10	0	1	0
15\1	9	0	2	0
20\1	10	1	3	0
10\2	10	1	0	0
15\2	10	1	2	1
20\2	10	3	4	1
12 months				
C	15	0	1	0
10\1	15	0	1	0
15\1	14	2	1	0
20\1	14	3	1	0
10\2	15	1	0	0
15\2	15	1	2	1
20\2	14	4	2	1

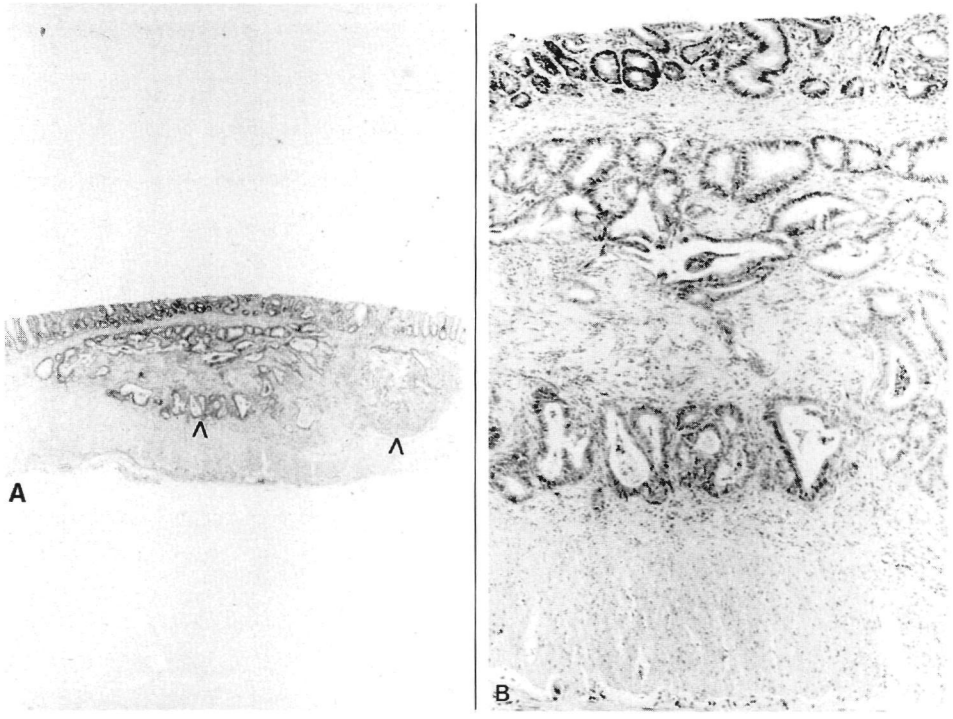

Figure VII-2 A,B. Photomicrograph with overview (**A**) and detail (**B**) of the colon of a rat (group 20/2, 6 months), showing 2 well-differentiated adenocarcinomas (arrows) in the submucosal layer of the irradiated segment.

The construction of the anastomosis had strongly increased both content and concentration in the anastomotic segment as compared to normal bowel, but, except for a difference in the hydroxyproline content in the 20/2 group at 6 months after the operation ($P<0.05$), there were no significant radiation effects found as compared to the control group.

In the colon distal of the anastomotic segment, the hydroxyproline concentration was increased in the 10/1 and 15/1 groups with respect to non-irradiated colon at 12 months after operation ($P<0.05$; two-tailed Mann-Whitney U test, Figure VII-4). In contrast to the anastomotic segments at 6 and 12 months after operation, there was no time-related increase of the hydroxyproline concentration or content from 6 to 12 months after operation.

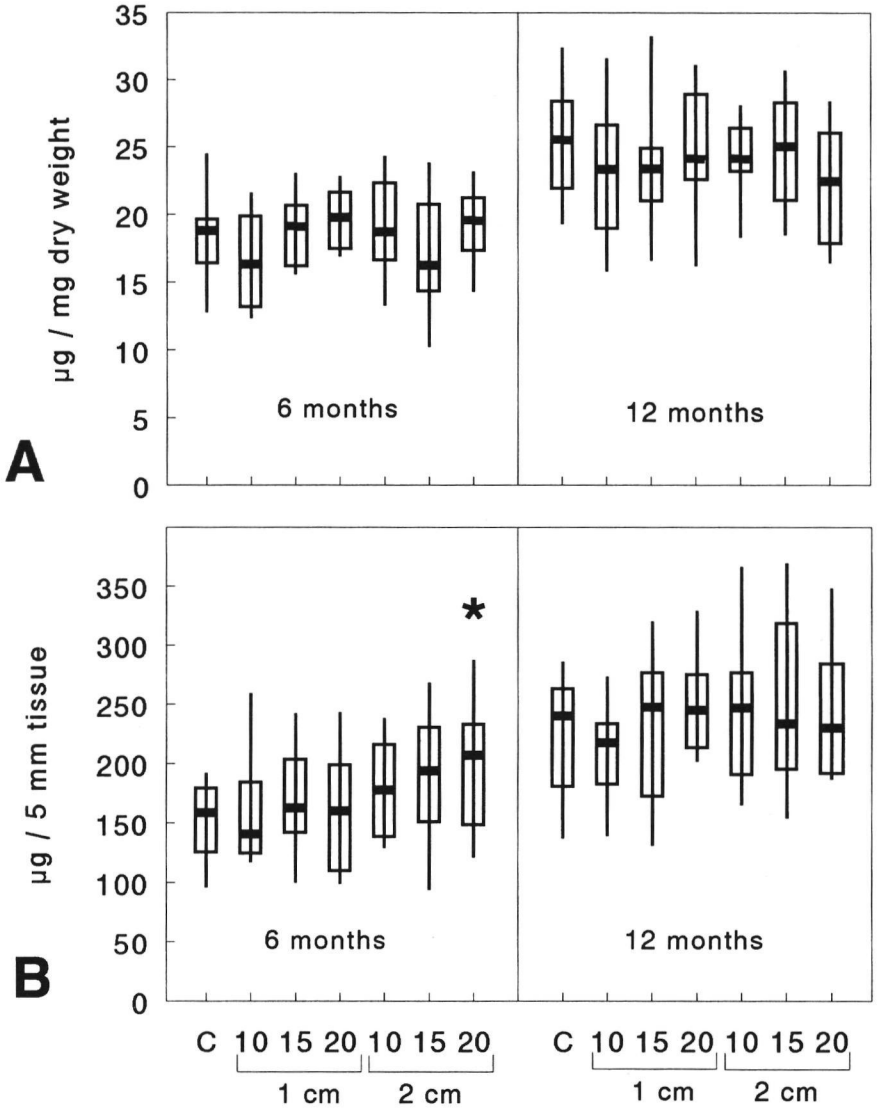

Figure VII-3A,B. Hydroxyproline concentration (**A**) and content (**B**) of anastomotic segments. The median value, 25-75% confidence interval (box), and 5-95% confidence interval are shown.
*: $P<0.05$ (2-tailed Mann-Whitney U test) difference vs. the control group.

DISCUSSION

In the present study we investigated dose-, time- and volume-dependent late

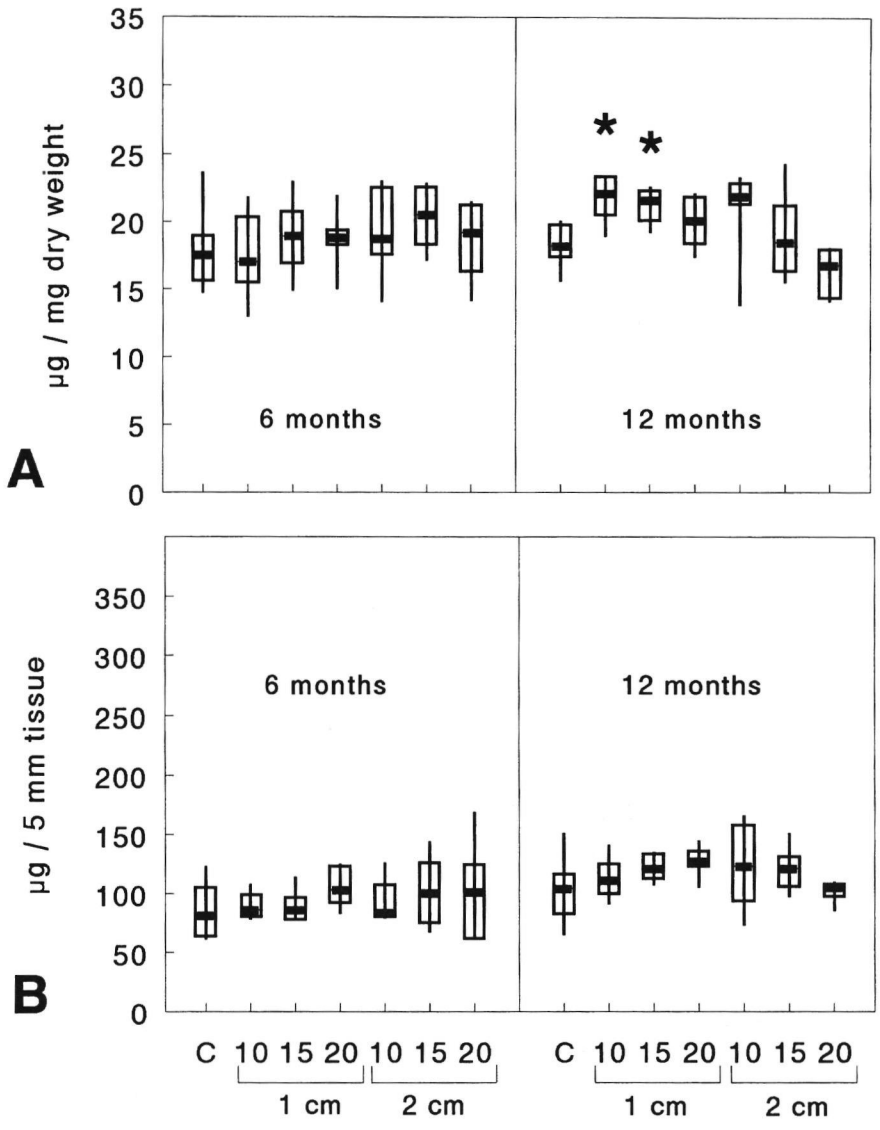

Figure VII-4A,B. Hydroxyproline concentration (**A**) and content (**B**) of distal (irradiated) segments. The median value, 25-75% confidence interval (box), and 5-95% confidence interval are shown.
*: $P<0.05$ (2-tailed Mann-Whitney U test) difference vs. the control group.

histological changes of colonic anastomoses due to intra-operative irradiation. Anastomotic wound healing was uneventful. Systematic analysis of six dose/volume radiation-related changes demonstrated that two

radiation-related effects (the formation of peri-anastomotic fibrosis and adenocarcinoma) were present at 6 and 12 months after irradiation and anastomotic construction. Both changes seemed to be dose-related; irradiation with a dosis of 20 Gy evoked most fibrosis and adenocarcinomas while 10 Gy had hardly any effect.

Mucosal ulcerations, often seen as a late side effect of irradiation for abdominal or pelvic tumors (15), were not found. While ulcerations are considered to be the result of ischemic damage and subsequent necrosis, the present results suggest that radiation-induced cell necrosis was too limited to lead to intestinal ulcerations. Rectal stenosis is often seen as a late complication of radiation therapy (11). In earlier experiments we have found functional rectal obstruction (proximal dilatation with fecal stasis, with normal bowel wall diameter at the site of the anastomosis) after the combination of irradiation with a dosis of 25 Gy and postoperative high-dose 5-fluorouracil (16). However, this was a study emphasising the short-term side effects of the combination of radiation and chemotherapy. In another study, monotherapy with radiation dose of 25 Gy did not lead to these side effects shortly after the operation (17). In a rat study, without anastomotic construction, Kiszel found that local irradiation of the large bowel could lead to fatal stenosis within 200 days (11). There was a steep rise of the incidence with dose. He determined the LD_{50} for a single radiation dose to be 20 Gy. The length of the irradiated volume was 2.4 cm, similar with the 2 cm group in our study. We could not confirm these findings: there was no difference in bowel diameter between control and experimental groups. Possibly the dose rate of the irradiation is of importance in this study (11). The dose rate is an important factor in cell-survival in normal and tumorous tissues (18). The influence of the dose rate on experimental colorectal tissue has been confirmed by Armour *et al.*, who determined the ED_{50} for rectal obstruction in the rat to be as high as 70.6 Gy if a low-dose-rate brachytherapy of 0.75 Gy/h was used (12).

Irradiation did not consequently lead to increased hydroxyproline (as a measure for collagen) levels in the anastomotic or distal segments. The surgical wounding itself led to an increase in collagen accumulation in the anastomotic segment, as is shown by the increase of the hydroxyproline content in the control animals between 6 and 12 months after operation (as well as in all irradiated rats). The distal parts showed no hydroxyproline

increase between 6 and 12 months post-irradiation. This is consistent with an earlier study in mice. Murray showed that after irradiation of the colon, collagen and total protein synthesis and breakdown rates were increased at early times, and returned to control levels by 4 months (19). After one year the structure of collagen, but not its level, had changed, and this accounted for the late functional changes of the irradiated intestine with decreased compliance of the bowel wall.

Experimentally, tumor growth after previous irradiation of the rectum has been described before (13). Clinically, there have been observations of rectal cancer developing after previous irradiation too: in the treatment of cervical cancer, relatively large radiation doses have been used with concomitant radiation-induced damage of the rectum (20). This side effect was reported to result in a 1.2 times increased chance for adenocarcinoma of the rectum (21). Storm even found a relative risk for rectal cancer of 2.4 (95% CI = 1.1-4.6), and also reported that cancers attributable to radiation therapy tend to appear late (10 or more years after radiotherapy), with an elevated risk for more than 30 years (22). Increased incidence of rectal cancer has also been reported by others (23).

Our data indicate that tumors were indeed a late radiation-induced phenomenon: a) the radiation dose and field were well defined; b) an adequate latent period of 6 and 12 months from time of irradiation to the onset of tumor was present; c) the tumors were located in the irradiated bowel in all cases; d) there were no tumors in sham-irradiated control rats. From this we conclude that the tumors were indeed radiation-induced.

In conclusion, it can be stated that treatment of the rat colon with a single radiation dose leads to dose- and volume-dependent late changes in the irradiated area. However, the progress of anastomotic wound healing remains uneventful. The formation of adenocarcinomas in the irradiated field is of concern to future investigations of efficacy of irradiation in animals with experimental large bowel cancer.

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SUMMARY

Chapter 1 contains a general introduction. Firstly, an overview is given on aspects of normal and impaired anastomotic healing of the large bowel. Then this the surgical treatment of cancer of the colon and rectum is discussed. There seems to be a definitive role for adjuvant radiation treatment. Subsequently, some technical aspects concerning radiotherapy are described, and the aspects of radio-enhancement and radioprotection are discussed. Also, an overview is given concerning the clinical aspects of the combination of surgery and radiotherapy. After this, unwanted side effects on normal tissues are described, which may appear both early and late, and limit the application of radiation therapy. Finally, the aim of the studies, which are depicted in the next chapters, is introduced shortly.

In the experiments described in **chapter 2** a dose of radiation is applied before the operation. In clinical practice this sequence is often used. A pilot study showed that external irradiation, preceding the operation, did not always lead to accurate irradiation of the designated colonic segment, despite a relatively large radiation field of 2.2 x 2.2 cm^2; this was probably due to parts of the colon which were located outside the irradiaton field. For this reason it was decided to irradiate the colon under direct vision by a small laparotomy. The single radiation dose was 25 Gy. After 1, 3, 5 or 28 days a colonic resection was performed with construction of an anastomosis; the proximal segment consisted of non-irradiated tissue, while the distal segment had been irradiated. The healing parameters were measured after 3 days in all groups, and after 7 days in groups irradiated 5 or 28 days before the operation. Macroscopically, the most severe radiation effects were seen in groups irradiated 5 or 28 days before the operation. The bowel wall was thickened by edema formation as a sign of an acute radiation effect. However, there were no differences in the healing of the anastomoses; also, bursting pressure and breaking strength showed no statistically significant differences from animals in the control group. The hydroxyproline content of the anastomotic segments was not different either, except in the group irradiated 28 days preoperatively; here a significantly higher value was measured, which indicates at an increase of connective tissue formation. Thus, early anastomotic healing after preoperative irradiation with a single dose of 25 Gy is not disturbed.

Chapter 3 describes a study of anastomotic healing after a combination of preoperative irradiation and postoperative chemotherapy. For this purpose 5-fluorouracil (5-FU) was used, since this is the single most efficient drug in the treatment of bowel cancer. Earlier experiments have shown that intra-peritoneal administration of 5-FU in a dose of 20 mg/kg/day for 7 days leads to impaired colonic anastomotic healing. For this reason a smaller dose was taken, and a possible dose-effect relationship was studied by the use of two different doses: 12.5 and 17.5 mg/kg/day. The experimental setup was unchanged: irradiation with a dose of 25 Gy was performed 5 days preoperatively, and a colonic resection was performed. From the day of operation onwards until sacrifice (day 7 postoperatively), 5-FU was administered once a day intra-peritoneally. As a result, body weight was decreased significantly as compared to the control group. The combination of irradiation and high-dose chemotherapy led to functional rectal stenosis in 40% of the rats: here a proximal dilatation was found together with fecal impaction and a normal anastomotic diameter. This effect was not seen in rats receiving radiotherapy combined with low-dose 5-FU. Bursting pressure and breaking strength were not different except in rats receiving high dose 5-FU as a single treatment. The hydroxyproline content of the anastomotic segments was increased in all rats receiving a combination of radiation and 5-FU. Thus, the combination of preoperative irradiation and 5-FU does not lead to impaired early strength of colonic anastomoses; however, the combination with high-dose 5-FU may lead to other sequelae like functional rectal stenosis.

The radiation effect on tumors can be enhanced in many ways. One of the methods is the combined use of radiation therapy and hyperthermia. In **chapter 4** the effect of this combination on normal bowel tissue is described; rats were irradiated with a dose of 25 Gy according to the experimental protocol described in chapter 2. Subsequently, a local colonic wash-out was performed with water of a temperature of 44°C, for 30 minutes. In this way, the distal colon was treated with radiation and heat. After 5 days a colonic resection was performed; an anastomosis was made with normal bowel tissue for the proximal leg and irradiated, heated tissue for the distal leg. At this time point, it was shown that the combination of radiation and heat had induced macroscopical transmural necrosis of the treated bowel in some rats. Animals were sacrificed 3 or 7 days

postoperatively. In groups which had received the combined treatment there were 4 (n=20) covered perforations without signs of generalised peritonitis. Also, anastomotic bursting pressures were significantly reduced, and the breaking strength was reduced as well (not significantly). The hydroxyproline content of the anastomotic segments was increased. After 7 days there were no significant differences any more. The data indicate that the combined treatment with irradiation and heat has unwanted side effects, while single treatment with radiation or heat has no short-term unwanted side effects on early anastomotic healing.

In **chapter 5** experimental anastomotic healing is further evaluated histologically. For this purpose, the experimental procedure as described in chapter 4 repeated. Five days after irradiation with a single dose of 25 Gy, and subsequent heat treatment with water of 44°C a colonic resection with construction of an anastomosis was performed. Animals were sacrificed 3 or 7 days later. The anastomotic segments were longitudinally cut in half; one part was used for histological slides which were stained with hematoxillin-eosin (HE) or Sirius Red. The other half was frozen and stained with a cell-specific antigen-antibody for macrophages (ED3). The slides stained with HE were studied for inflammation, necrosis, and impaired anastomotic healing, while in the Sirius Red stained slides a semiquantative measurement of the amount of collagen fibers in the anastomotic area was performed. In animals treated with the combination therapy, obvious necrosis of the bowel wall was observed, which was consistent with results of the experiment described in chapter 4. Also, a high incidence of anastomotic healing disturbances was observed. There was enhanced infiltration with polymorphonuclear leucocytes. Animals receiving single treatment and sham-treated animals in the control group showed relatively unhampered anastomotic healing. Semiquantitative collagen measurements, and lymphocyte or macrophage counts in the anastomotic area showed no differences between groups either. The results in this histological study support the findings of the experiment described in chapter 4.

Chapter 6 contains a study after the effects of both intra-operative and postoperative irradiation on early anastomotic repair. A single radiation dose of 20 or 25 Gy was used directly after colonic resection and construction of

an anastomosis. There was no difference in bursting or breaking strength of the anastomosis 3 or 7 days postoperatively. If the skin was closed before the application of radiotherapy (direct post-operative external irradiation), a volume effect was shown. In this group more intestinal tissue was irradiated because of an increase of the irradiated field; the body weight of the rats decreased markedly, and there was a significant fall in the serum albumin and protein level, despite the relatively low radiation dose of 20 Gy. This study shows that intra-operative radiotherapy can be an adjuvant treatment without serious side effects on early anastomotic healing in an experimental model.

Finally, in **chapter 7** long-term healing of colonic anastomoses following intra-operative irradiation is described, and a radiation dose and volume effect are investigated. In this study a single radiation dose of 10, 15 or 20 Gy was applied intra-operatively. The anastomosis was constructed using untreated tissue for the proximal leg and irradiated tissue (over a length of 1 or 2 cm) for the distal leg. Body weight of the animals was measured every month; the animals were killed after 6 or 12 months. After macroscopical inspection, the anastomotic segment was excised, and this specimen was longitudinally cut in half. One part was used to prepare microscopic slides which were stained with hematoxilin-eosin (HE) or Sirius Red, while in the other half the hydroxyproline concentration and content were measured in a 5 mm segment containig the anastomosis, and in the adjacent distal segment. Macroscopic examination revealed some signs of late radiation effects: there were mild peri-anastomotic adhesions in some of the irradiated rats. There was no difference in bowel diameter. Histological examination revealed adenocarcinomas in 11 rats irradiated with a dose of 20 Gy (n=48), in 4 rats after a dose of 15 Gy (n=48) and in 2 rats after a dose of 10 Gy (n=50). Control rats (n=25) developed no malignancies. Most malignancies developed after 12 months (11/17), and after irradiation of 2 cm bowel (11/17). Furthermore, there was some dose-related formation of fibrosis in irradiated animals. In all groups, the anastomotic hydroxyproline content increased between 6 and 12 months postoperatively. However, there were no significant differences between groups. Thus, intra-operative irradiation of one anastomotic leg evokes dose-related changes (adenocarcinomas and fibrosis), and time- and volume-related changes (adenocarcinoma). Still, anastomotic healing is unimpaired.

In **hoofdstuk 1** wordt een algemene inleiding gegeven. Allereerst wordt een overzicht gegeven van aspecten van de normale en verstoorde naadgenezing van de dikke darm. Hierna wordt ingegaan op de chirurgische behandeling van het carcinoom van colon en rectum. Er lijkt een duidelijke plaats te zijn voor adjuvante behandeling met radiotherapie. Vervolgens worden enkele technische aspecten van radiotherapie behandeld en wordt gekeken naar agentia en methoden om de effectiviteit van straling te vergroten of te beperken. Ook wordt een overzicht gegeven van de klinische aspecten van een combinatie van chirurgie en bestraling. Hierna worden ongewenste neveneffecten van straling op normale weefsels beschreven. Deze treden zowel in een vroege als in een late fase op en beperken het gebruik van radiotherapie. Uiteindelijk worden de doelen van de studies, welke in de volgende hoofdstukken worden beschreven, kort ingeleid.

In **hoofdstuk 2** wordt de bestraling vóór de operatie toegepast. In de dagelijkse praktijk is dit een veel gebruikte volgorde. Een pilot studie toonde aan dat bij een uitwendige bestraling voorafgaand aan de operatie, bij een bestralingsveld van 2,2 x 2,2 cm^2, het niet altijd zeker is dat het beoogde colon-gedeelte ook inderdaad wordt bestraald; gedeelten van de te bestralen darm kunnen ook buiten het bestralingsveld liggen. Om deze reden werd gekozen voor het operatief vrij leggen van het te bestralen gedeelte via een laparotomie. De eenmalige dosis bedroeg 25 Gy. Na 1, 3, 5 of 28 dagen werd vervolgens een colonresectie verricht en een anastomose gemaakt. Het proximale segment van de anastomose bestond uit niet-bestraald weefsel, het distale segment was wel bestraald. De naadgenezing werd in alle groepen 3 dagen na operatie gemeten, en in 2 groepen (respectievelijk 5 en 28 dagen na preoperatieve bestraling) 7 dagen na operatie gemeten. Macroscopisch werden de grootste effecten gezien wanneer 5 of 28 dagen tevoren was bestraald. De darmwand was verdikt door oedeemvorming als teken van een acuut stralingseffect. Er waren geen verschillen in genezing van de anastomosen; barststerkte en treksterkte waren statistisch niet significant verschillend ten opzichte van de waarden bij dieren uit de controle groep. Het hydroxyproline-gehalte van de anastomose was eveneens niet verschillend, behalve in de groep die 28 dagen preoperatief was bestraald. Hier werd een significant hogere waarde gevonden, hetgeen wijst op een versterkte bindweefsel-vorming. In conclusie kan gesteld

worden, dat vroege naadgenezing na preoperatieve bestraling met een eenmalige dosis van 25 Gy ongecompliceerd verloopt.

In **hoofdstuk 3** wordt de darmnaad-genezing na een combinatie van preoperatieve bestraling met postoperatieve chemotherapie beschreven. Hiervoor werd 5-fluorouracil (5-FU) gebruikt, het meest effectieve chemotherapeuticum bij de behandeling van patiënten met dikke-darmkanker. Uit eerdere experimenten is bekend dat de toevoeging van 5-FU in een dosering van 20 mg/kg/dag gedurende 7 dagen leidt tot verslechtering van darmnaad-genezing. Om deze reden werd gekozen voor een lagere dosering. Een dosis-effect relatie werd onderzocht door het effect van zowel 12,5 als 17,5 mg/kg/dag te meten. Het onderzoeksprotocol was gebaseerd op dat van het voorafgaande experiment: 5 dagen preoperatief werd bestraald met een dosis van 25 Gy, en er werd een colonresectie verricht. Vanaf dit moment tot de dag van opoffering - dag 7 - werd 5-FU dagelijks intraperitoneaal toegediend. Op het moment van opofferen was het lichaamsgewicht van de ratten significant lager dan in de controle groep. De combinatie van straling en hoge dosis 5-FU leidde tot een functionele rectum stenose bij 40% van de ratten: hierbij was er sprake van een proximale dilatatie van de darm met faecale impactie, doch met een normale diameter van de anastomose. Dit effect werd niet gezien bij de combinatie met lage dosis 5-FU. De barststerkte en de treksterkte van de darmsegmenten met de anastomose waren niet verschillend behalve in de groep welke alleen hoge dosis 5-FU kreeg toegediend. Het hydroxyproline-gehalte in de naadsegmenten was in beide groepen die behandeld waren met bestraling en 5-FU verhoogd. De conclusie is dat de vroege wondgenezing na een combinatie van preoperatieve bestraling en 5-FU niet leidt tot vermindering van de sterkte van anastomoses in de vroege fase van de wondgenezing, hoewel de combinatie met een hoge dosis 5-FU toch mogelijk tot afwijkingen kan leiden, zoals een functionele rectum stenose.

Het effect van bestraling op tumoren kan op velerlei wijze worden versterkt. Een van de manieren is de combinatie van bestraling en locale hyperthermie. In **hoofdstuk 4** zijn de effecten van deze combinatie op normaal darmweefsel onderzocht; ook nu werden ratten preoperatief bestraald met een dosis van 25 Gy volgens de techniek uit hoofdstuk 2.

Direct aansluitend werd een locale darmspoeling gegeven met water met een temperatuur van 44°C gedurende een half uur. Hierdoor werd het laatste stukje colon dus zowel met straling als met warmte behandeld. Vijf dagen later volgde een colonresectie waarbij ervoor gezorgd werd dat het distale segment van de anastomose bestond uit behandelde darm, terwijl het proximale segment bestond uit onbehandelde darm. Tijdens de operatie, 5 dagen na de initiële behandeling, werden na de combinatie van bestraling met hyperthermie macroscopische transmurale necrose plekken in de behandelde darm van sommige ratten waargenomen. De dieren werden opgeofferd na 3 of 7 dagen. Bij de groepen behandeld met zowel straling als warmte waren er vier (n=20) afgedekte perforaties zonder tekenen van gegeneraliseerde buikvliesontsteking; verder was de barststerkte na 3 dagen significant verlaagd en ook de treksterkte was lager (niet significant). Na 7 dagen waren er geen verschillen meer. Wel was het hydroxyproline-gehalte van het darmsegment met de anastomose significant verhoogd. Er kan worden geconcludeerd dat de gecombineerde behandeling met straling en warmte schadelijke bijwerkingen heeft op de vroege darmnaadgenezing, terwijl de combinatie van operatie met alleen straling of warmte geen bijwerkingen heeft op korte termijn.

In **hoofdstuk 5** wordt de genezing van experimentele anastomoses verder histologisch geëvalueerd. Voor dit onderzoek werd de opzet van het experiment uit hoofdstuk 4 gehanteerd. Na preoperatieve bestraling met een eenmalige dosis van 25 Gy, en warmte-behandeling door middel van een locale darmspoeling met water van 44°C, volgde na 5 dagen een darmresectie met het aanleggen van een anastomose en opofferen 3 of 7 dagen later. Het stuk darm met de anastomose werd longitudinaal in tweeën gedeeld; van de ene helft werden histologische coupes gemaakt die werden gekleurd met hematoxilline-eosine (HE) of Sirius Rood. Van de andere helft werd op vriescoupe een immuunhistologische kleuring van macrofagen gemaakt door middel van celspecifieke antigeen-antilichaam-kleuring (ED3). In de coupes met HE kleuring werd gekeken naar ontstekingseffecten, necrose en al of niet verstoorde darmnaad-genezing, terwijl in de Sirius Rood coupes een semi-kwantitatieve meting werd verricht van de hoeveelheid collageenvezels in het anastomose gebied.
Bij dieren die behandeld waren met de combinatie radiotherapie en

hyperthermie werd, net als in het experiment uit hoofdstuk 4, duidelijke necrosevorming waargenomen en was er sprake van een hoge frequentie van naadgenezingsstoringen. Er was toegenomen infiltratie met polymorfkernige leucocyten. Bij dieren die alleen bestraald werden of die alleen hyperthermie kregen en dieren in de controle groep verliep de naadgenezing normaal. De semi-kwantitatieve collageenmeting liet geen verschillen zien tussen groepen en hetzelfde gold voor de lymfocyten- en macrofagen-infiltratie rond de anastomose. De conclusies uit hoofdstuk 4 konden met de resultaten van dit histologisch onderzoek worden ondersteund.

In **hoofdstuk 6** wordt het effect bekeken van de invloed van intra-operatieve en postoperatieve bestraling op de vroege darmnaadgenezing bij ratten. De gebruikte eenmalige dosis was 20 of 25 Gy, welke direct na de colonresectie en het aanleggen van een anastomose werd toegepast. Zowel 3 als 7 dagen na het aanleggen van de naad was er geen verschil in barst- of treksterkte van de anastomose. Anders verliep de genezing indien eerst de huid gesloten werd en direct daarna een uitwendige bestraling werd toegepast. Hier kwam duidelijk het volume-effect van de bestraling naar voren: door een iets groter veld werd een grotere hoeveelheid darmweefsel bestraald; de ratten verloren veel gewicht en vertoonden een ernstige daling van het albumine- en eiwitgehalte in het serum, ondanks de relatief lage stralingsdosis van 20 Gy. Deze studie toont aan dat intra-operatieve bestraling een veilige adjuvante behandeling kan zijn wat betreft de neveneffecten op de vroege naadgenezing in een experimenteel model.

Hoofdstuk 7 tenslotte behandelt de effecten van intra-operatieve bestraling op darmnaadgenezing op lange termijn, waarbij tevens een volume effect werd onderzocht. Er werd een intraoperatieve bestraling toegepast met een eenmalige dosis van 10, 15 of 20 Gy. Het volume effect werd onderzocht door in elke groep na resectie van een colonsegment het distale darmsegment te bestralen over een lengte van 1 of 2 cm. Hierna werd een anastomose gemaakt. Het gewicht van de dieren werd maandelijks gemeten. Opoffering volgde na 6 of 12 maanden. Na macroscopische inspectie werden de naadsegmenten longitudinaal in tweeën verdeeld; van de ene helft werden coupes gemaakt welke werden gekleurd met hematoxilline-eosine (HE) of Sirius Rood, terwijl in de andere helft de hoeveelheid

hydroxyproline werd gemeten in zowel het anastomose gedeelte als in het bestraalde (distale) gedeelte. Bij macroscopische inspectie was er sprake van enige late stralingsreacties: er waren matige adhaesies rond de anastomose in sommige bestraalde ratten. Er was geen verschil in diameter van de darm. Histologisch onderzoek toonde de vorming aan van adenocarcinomen bij 11 van de 48 ratten na bestraling met 20 Gy, bij 4 van de 48 ratten na bestraling met 15 Gy en bij 2 van de 50 ratten na bestraling met 10 Gy. In de controle groep trad dit verschijnsel niet op (0/25). De meeste carcinomen werden gevonden in de groepen die 12 maanden tevoren waren bestraald (11/17), en na bestraling van 2 cm darm (11/17). Ook nam na bestraling de hoeveelheid fibrose rond de anastomose toe; dit effect was afhankelijk van de stralingsdosis. In alle groepen werd een toename gezien van de hoeveelheid hydroxyproline tussen 6 en 12 maanden na de operatie. Er waren echter geen significante verschillen tussen de groepen onderling. De conclusie is dat intra-operatieve bestraling op lange termijn stralingsdosis-gerelateerde veranderingen (fibrosis en de vorming van adenocarcinomen) en volume- en tijd-gerelateerde veranderingen (vorming van adeno-carcinomen) tot gevolg heeft. De naadgenezing verloopt echter ongestoord.

DANKWOORD

Het proefschrift wat voor u ligt kon slechts tot stand komen dankzij de medewerking van een groot aantal personen. Zij hebben allen in meer of mindere mate een bijgedrage geleverd door hun inzet, inzicht, analyse, hulp, discussie, vragen, interesse of gewoon door hun betrokkenheid.

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medewerkers van de afdeling medische statistiek
medewerkers van de afdeling radiodiagnostiek

CURRICULUM VITAE

De auteur van dit proefschrift werd op 28 september 1958 geboren te Rotterdam.
In Dordrecht werd in 1976 het eindexamen Atheneum-B gehaald. Van 1977 tot 1984 volgde hij de medische studie aan de Rijks Universiteit te Utrecht. Aansluitend was hij tot 1 februari 1986 werkzaam als AGNIO chirurgie in het Gemeente Ziekenhuis te Hilversum. Nadat hij, via de centrale selectie, met succes had meegedongen naar een opleidingsplaats heelkunde, werden de eerste 3 jaren van de opleiding gevolgd in het Gemeente Ziekenhuis te Arnhem onder leiding van dr. W.F. Eggink. De laatste 3 jaren van de opleiding werden gevolgd in het Academisch Ziekenhuis Nijmegen St. Radboud, onder leiding van Prof. dr. R.J.A. Goris.
Sinds 1 februari 1992 is de auteur werkzaam in het Academisch Ziekenhuis Nijmegen St. Radboud als chirurg met als aandachtsgebied traumatologie. Van 1 april 1993 tot 1 april 1995 werd hier tevens de vervolgopleiding traumatologie gevolgd (CHIVO), onder leiding van Prof. dr. C.J. van der Linden.
In 1992 trouwde hij met Annemieke de Vries. Zij hebben 3 kinderen: Jan (1993), Marjolein (1995), en Emmelien (1996).

Stellingen behorende bij het proefschrift

IRRADIATION AND HEALING OF COLONIC ANASTOMOSES

an experimental study in the rat

1. Experimenteel onderzoek naar bestraling en darmnaad-genezing is goed mogelijk in een rattenmodel *(dit proefschrift)*.

2. Na pre- of intra-operatieve bestraling verloopt experimentele naadgenezing van het colon bij de rat op korte termijn ongestoord, ook bij een relatief hoge stralingsdosering *(dit proefschrift)*.

3. De combinatie van behandeling met preoperatieve bestraling en hyperthermie kan bij ratten aanleiding geven tot ernstige necrose van het colon *(dit proefschrift)*.

4. Bij de combinatie van behandeling met preoperatieve bestraling en postoperatieve chemotherapie bij de rat treedt een dosis-effect relatie op; ongestoorde naadgenezing van het colon is mogelijk *(dit proefschrift)*.

5. Intra-operatieve bestraling van het colon van de rat kan op lange termijn leiden tot de vorming van adenocarcinomen *(dit proefschrift)*.

6. Bij de meeste calcaneusfracturen leidt de minimaal invasieve behandeling door percutane repositie en gecannuleerde schroeffixatie tot een goed functioneel resultaat op lange termijn.

7. Wielrenners dienen te beseffen dat de zogenaamde "clipless pedalen", naar analogie van skibindingen, correct dienen te worden afgesteld, teneinde ernstig letsel te voorkomen (*Ned Tijdschr Geneesk 1995; 139:1141-3*).

8. Uit oogpunt van verkeersveiligheid verdient het de voorkeur om vóór de bomenrijen, welke ter landschappelijke verfraaiing langs de kant van wegen buiten de bebouwde kom zijn geplaatst, een vangrail te plaatsen, danwel deze bomen te rooien.

9. Ongevalsletsels waardoor mensen dood gaan zijn meestal niet de letsels waardoor mensen invalide raken, en andersom.

10. De afkorting R.I.P. voor ruimte innemend proces dient te worden vermeden - dit geeft een verkeerde indruk van de stand van zake bij de hedendaagse kankerbestrijding.

11. "Adventure"-vakanties, "survival"-weekenden en klim-expedities dienen niet via een reisbureau geboekt te worden.

12. Telefonische enquetes zorgen ervoor dat je gestoord wordt.

13. Mensen met een piercing van een deel van het gezicht hebben een gaatje in hun kop.

Jan Biert
Heilig Landstichting, 24 juni 1997