The widespread success of microvascular free tissue transfer has enabled more aggressive tumor resection while regaining form and function necessary for acceptable quality of life. Malignant tumor treatments are based on a multidisciplinary approach that generally requires the combination of wide surgical margins, radiotherapy, and chemotherapy.1–3 Radiation therapy is used against many types of cancer. About 60% of cancer cases require radiation therapy.5 The usual radiation protocols are preoperative or postoperative external beam treatment, or brachytherapy.4,6–9 The advantages of neoadjuvant radiotherapy include smaller field sizes,7,10 lower doses,11,12 and radiation-induced tumor regression, which may reduce the extent of surgical resection and spare critical structures.4,6–8,13,14

Despite potential advantages of neoadjuvant radiotherapy, it is not universally practiced, especially because several studies have identified preoperative radiotherapy as an independent risk factor for complications after microvascular reconstruction,1,7,12 such as wound infection, flap loss, delayed wound healing, and prolonged hospitalization.15–25 Success rates of microsurgery procedures in non-

Effect of Previous Irradiation on Vascular Thrombosis of Microsurgical Anastomosis: A Preclinical Study in Rats

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Background: The objective of the present investigation was to compare the effect of neoadjuvant irradiation on the microvascular anastomosis in cervical bundle using an experimental model in rats.

Methods: One hundred forty male Sprague–Dawley rats were allocated into 4 groups: group I, control, arterial microanastomosis; group II, control, venous microanastomosis; group III, arterial microanastomosis with previous irradiation (20 Gy); and group IV, venous microanastomosis with previous irradiation (20 Gy). Clinical parameters, technical values of anastomosis, patency, and histopathological parameters were evaluated.

Results: Irradiated groups (III and IV) and vein anastomosis groups (II and IV) showed significantly increased technical difficulties. Group IV showed significantly reduced patency rates (7/35) when compared with the control group (0/35). Radiotherapy significantly decreased the patency rates of the vein (7/35) when compared with the artery (1/35). Groups III and IV showed significantly reduced number of endothelial cells and also showed the presence of intimal thickening and adventitial fibrosis as compared with the control group.

Conclusion: Neoadjuvant radiotherapy reduces the viability of the venous anastomosis in a preclinical rat model with a significant increase in the incidence of vein thrombosis. (Plast Reconstr Surg Glob Open 2016;4:e1073; doi: 10.1097/GOX.0000000000001073; Published online 28 November 2016.)
Irradiated tissues range from 90% to 99% depending on the procedure. Whether these results are the same in previously irradiated tissues remains controversial. Microvascular surgical techniques in a previously irradiated field are technically demanding, with increased incidence of postoperative complications. Currently, it has been debated whether radiation has an effect on free flap survival. The existing clinical data on free flap reconstruction after radiotherapy focus primarily on wound-related complications and average flap survival without reporting the rate of thrombosis of the vascular microanastomosis. It is widely agreed that the majority of microsurgical failures are due to vascular phenomena such as arterial thrombosis, venous thrombosis, or both. Thrombosis is defined as the formation of a clot inside a blood vessel. It has been described that microvascular compromise occurs due to venous thrombosis leading to flap congestion and necrosis rather than to arterial thrombosis. In addition to technical difficulties in the execution of the microanastomosis because of the thin wall of the veins, the slow blood flow due to low blood pressure in the venous system contributes to thrombus formation.

To date, there is a paucity of reliable studies that have specifically addressed the effect of prior radiation therapy on the rate and specific type of microvascular alterations after vascular anastomosis. There is also lack of consensus in previous experimental studies. Moreover, these studies are not recent and are bereft of statistical evidence. Thus, the underlying vascular pathology remains unclear. This experimental study was performed to define the thrombotic effect of preoperative irradiation on vascular anastomoses.

**MATERIALS AND METHODS**

The ethics committees of Vall d’Hebron Research Institute and Hospital Universitari Vall d’Hebron (Universitat Autònoma de Barcelona) approved the experimental protocol. All animals received care in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and the Guide for Care and Use of Laboratory Animals prepared by the National Institutes of Health (publication number, 80-23, revised 1985) and the Spanish law of protection of experimental animals (Real Decreto 223, 1988).

One hundred forty adult male Sprague–Dawley rats, weighing on average 300 g (range, 250–400 g), were obtained from Janvier Labs (Roubaix, France). The animals were divided into 4 groups of 35 rats each: group I, control anastomosis in the common carotid artery without previous irradiation; group II, control anastomosis in the external jugular vein without previous irradiation; group III, anastomosis in the common carotid artery with previous irradiation; and group IV, anastomosis in the external jugular vein with previous irradiation (Fig. 1). All animals were followed up after surgery for 6 weeks, when the end point was established according to previous animal studies to be able to recognize acute and subacute thrombosis.

**Irradiation Technique**

Computed tomographic images were used as a basis for dose planning before the irradiation (Fig. 2). Radiotherapy was given 14 days before the operation in groups III and IV. The time lapse between the radiotherapy and the day of the surgery was established following previous animal models, which demonstrated that surgery carried out between the second and tenth days is still dangerous because of hypervascularity stage of the tissues, and therefore surgery should be done between 2 and 6 weeks after irradiation in the hypovascular stage of tissue. The rats in the irradiated groups were exposed in subgroups of 4 rats simultaneously to a single fraction of 20 Gy, delivered at a mean dose rate of 1.0 Gy per minute.

Fig. 1. Flowchart of the experimental study. Time lapse between the radiotherapy (RT) and the day of the surgery and time tracking are detailed. FU, follow-up.
at an 200-mm source-to-skin distance. A central lead plate allowed simultaneous irradiation of 4 fields. The radiation was applied to the left hemicervical region after anesthesia with ketamine hydrochloride (50 mg/kg; Ketalar, Parke Davis, Eczacıbaşı, Istanbul, Turkey) and xylazine (5 mg/kg). A midline cervical approach was performed and the carotid artery or jugular vein was dissected and isolated with the aid of a microscope at 10× magnification. We measured the cervical vessel diameters at this point with a standardized background measuring grid before extensive dissection/stripping. The vessels were occluded with clamps (P-2, S&T, Neuhausen, Switzerland) and sectioned. Standardized end-to-end microvascular anastomosis was performed at 20× magnification with interrupted stitches of 9.0 nylon suture with a 50-μm needle (9.0 monofilament polypropylene, Prolene, Ethicon, Weymouth, United Kingdom). We measured the time needed to perform the microvascular anastomosis (minutes), the number of stitches for every anastomosis, and the number of attempts for a successful anastomosis. Subsequently, the clamps were removed to allow recovery of the blood flow, and at this moment we measured the time to achieve the hemostasis of the anastomotic site (seconds). Patency of the microvascular anastomosis was assessed by visual observation and a patency test at 1, 3, and 5 minutes after the procedure. The empty and refill patency test should be performed gently. Two pairs of smooth forceps are used to occlude the vessel distal to the anastomosis. The more “downstream” forceps is then moved gently approximately 1 cm down the vessel to create an empty segment between the 2 forceps. The proximal compression is then released, and rapid filling of the empty segment indicates patency of the anastomosis. This test is useful for either arteries or veins and for any size of vessel. The approach was closed with interrupted 5.0 nylon suture (monofilament polypropylene, Prolene, Ethicon, Weymouth, United Kingdom). Buprenorphine was administered subcutaneously as an analgesic with a dose of 0.01 mg/kg twice daily after the surgical procedure to all animal subjects.

All animals were surgically explored 6 weeks after the microsurgery under the same anesthesia protocol used in the first surgery. Patency of both common carotid arteries (left and right) and both external jugular veins (left and right) at the time of sacrifice was assessed by visual observation and patency test. This variable was used to define thrombosis macroscopically. Direct inspection of both arterial and venous anastomoses under the microscope may reveal signs of patency. Arterial patency is performed by nicely dilating vessels showing pulsatile elongation (“wriggling”) or expansile pulsation. Gently lifting the vessel distal to the anastomosis by placing forceps underneath will demonstrate the “flicker” of blood flowing across this area, but it is easily visible only in thin-walled vessels.

Specimens, including the anastomosed site, were taken for histopathological study. All biopsies were preserved in formalin solution, and then stained with hematoxylin–eosin (H&E) and Masson’s trichrome and evaluated with light microscopy by the same pathologist.
Histological Analysis

A conventional binocular Zeiss (Carl Zeiss Microscopy, Jena, Germany) light microscope was used with an ocular magnification of 10× and objectives 25/0.45 and 40/0.65 for examination of the H&E-stained sections. All observations were performed by 1 examiner (C.R.), who was unaware of the origin of the slides. A semiquantitative scale adapted from Verhofstad et al47 and containing various parameters for analysis of the wound healing sequence was used (Table 1). The amount of necrosis was expressed as none (0 point), only small patches (1 point), some patches (2 points), or massive (3 points). In the anastomotic area, accumulation of polymorphonuclear cells, macrophages, and lymphocytes was assessed in terms of none or normal number (0 point), slight increase (1 point), marked infiltration (2 points), or massive infiltration (3 points). Edema, expressed as the ratio of maximum thickness of the wall at the anastomosis to the thickness of the normal vascular wall as present at the end of the section, was established in terms of none (0 point), some (1–1.5× normal thickness; 1 point), marked (1.5–2× normal thickness; 2 points), or severe (>2× normal thickness; 3 points). Healing of the anastomotic site was expressed as normal, that is, endothelial layer with restored simple endothelial cells (0 point), small patches without endothelial cells (1 point), large patches without endothelial cells (2 points), or endothelial completely devoid of endothelial cell coverage (3 points). Intimal layer and muscular repair was assessed in terms of good (0 point), average (1 point), poor (2 points), or no (3 points) fibroblast stretching and bridging the anastomotic wound.

Statistical Analysis

Statistical analysis was performed using SPSS Statistics, Windows version 18.0 (SPSS Inc., Chicago, Ill.). Data were expressed as mean ± SD if normally distributed or as median and range. For all tests, \( P < 0.05 \) was considered significant. Results were analyzed through Kruskal–Wallis variance analysis. Mann–Whitney test was used to evaluate the differences between the mean of both groups. Chi-square analysis was used with categorical variables and the \( t \) test was used with continuous variables to assess for univariate predictors of thrombosis after a microanastomosis.

RESULTS

Survival Rate

Groups I and II: All rats survived the 6-week study period 100%. Group III: 97% survival rate, with 1 rat lost
during the acute period post-radiotherapy. Group IV: 97% survival rate, with 1 rat was lost during the subacute period post-radiotherapy (2–4 weeks). Statistically significant differences were not observed between groups ($P > 0.05$).

**Weight**

Results are depicted in Figure 4. There was a progressive increase in body weight in control groups (I and II) and the irradiated groups (III and IV) until the conclusion of the study. No significant differences were observed between groups ($P > 0.05$).

**Vascular Patency and Macroscopic Appearance**

Patency was 100% in the control groups (groups I and II), whereas it was 97.1% in group III (only 1 patency test was negative) and 80% in group IV (7 patent tests were negative; (Fig. 5). The patency rates of the arterial radiotherapy group (group III) were not statistically different from those of the control arteries (group I; $\chi^2$ test, $P > 0.05$). The patency rates of the venous radiotherapy group (group IV) were statistically significantly different from those of the control veins (group II; $\chi^2$ test, $P < 0.05$). The patency rates of control arteries (group I) were not statistically different from those of the control veins (group II; $\chi^2$ test, $P > 0.05$). The patency rates of radiotherapy arteries (group III) were statistically significantly different from radiotherapy veins (group IV; $\chi^2$ test, $P < 0.05$).

Macroscopically, both irradiated and nonirradiated arteries and veins did not show surface or endovascular alterations visible through the surgical microscope.

**Anastomosis Technical Evaluation**

A correlation between the vascular patency and the technical difficulties in the execution of the anastomosis was performed. Results of the univariate analysis are summarized in Figure 6. This revealed 4 statistically significant variables that increased vascular thrombosis: time of anastomosis, number of stitches, number of attempts for a successful anastomosis, and time to achieve hemostasis. All the thrombosed vessels (8/140) needed more than 1 (2–3, range) anastomosis attempt to achieve an initial vascular patency success.

**Histological Study**

Statistically significant differences were not observed between groups I versus II (non-radiotherapy groups) and III versus IV (radiotherapy groups; $P > 0.05$).

In the radiotherapy groups, we found significant changes compared with the non-radiotherapy groups (Fig. 7): with moderate decrease in the number of endothelial cells, the intimal layer was thicker with moderate fibroblast proliferation (Fig. 8); the number of muscle cells in the media was slightly decreased and vacuolation was observed (Fig. 9).

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**Table 1. Parameters of Histological Study**

| Grade | Necrosis       | PML       | Mononuclear Cells | Edema     | Fibrosis | Cells Number |
|-------|----------------|-----------|-------------------|-----------|----------|--------------|
| 0     | None           | None      | None              | None      | None     | None         |
| 1     | Small patches  | Slight infiltration | Slight infiltration | Slight     | Slight   | Slight infiltration |
| 2     | Large patches  | Moderate infiltration | Moderate infiltration | Moderate   | Moderate | Moderate infiltration |
| 3     | Massive patches| Massive infiltration | Massive infiltration | Severe     | Severe   | Massive infiltration |

PML, polymorphonuclear leucocyte.

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**Fig. 4.** Data represent mean values of body weight on the day of the surgery and at 2, 4, and 6 weeks postoperative. Numbers represent $P$ values of the significant differences observed between groups.
Some histological changes were observed in both groups (non-radiotherapy vs radiotherapy groups) without significant differences between groups \( (P > 0.05) \), as the external elastic lamina was altered with fibrosis, microcalcifications, and necrotic fields around suture holes. There was also edema in the adventitia, and there was a slight infiltration of polymorphonuclear leucocysts in the medial layers and adventitia. These changes were considered due to surgical procedure.

The histological study confirmed the presence of a thrombus on the anastomotic site in all the cases where thrombosis was observed macroscopically or through a negative patency test.

**DISCUSSION**

Preoperative radiotherapy provoked histological changes in both arterial and venous microanastomoses but only resulted in an increased rate of venous thrombosis in our rat model.

Regarding the experimental literature, some previous models have attempted to examine radiation-induced changes on microvascular anastomoses (Table 2).\(^{31,32,41–43,45,46}\) Results obtained regarding patency have shown high variability. Furthermore, this patency variability increases with its association with radiotherapy.\(^{7,12,15,19–25}\) Some of these experimental studies concluded that acute irradiation may predispose to free flap failures.\(^{51,42,45,46}\) On the contrary, oth-
er studies suggest that radiation may not alter the patency of arteries but have detrimental effects only on veins. These studies are inconclusive due to uncontrolled biases or lack of statistical power. Many series were too small with an insufficient number of observations to provide a minimal risk of type II error. In some cases, their experimental
Table 2. Previous Experimental Models of Microanastomosis on Irradiated Population

| Study                | Animal/Strain       | n  | RT Type                                | Irradiation Zone | RT Dose | Surgical Procedure       | Results                                      |
|----------------------|---------------------|----|----------------------------------------|------------------|---------|--------------------------|----------------------------------------------|
| Baker et al\(^{41}\) | Rat Sprague–Dawley | 28 | Neoadjuvant                            | Groin            | 4 Gy    | Arterial anastomosis     | No statistically significant differences     |
|                      |                     |    | Single dose                            |                  | 6 Gy    | Femoral                   | Thrombosis RT 22.2%                          |
|                      |                     |    | 4wk pre SP                             |                  | Control group |                           | Thrombosis control 30%                        |
| Tan et al\(^{45}\)  | Rabbits New Zealand | 48 | Neoadjuvant                            | Groin            | 2 Gy    | Epigastic free flap to femoral vessels | Statistically significant differences 50% necrosis flaps RT |
|                      |                     |    | Single dose                            |                  | Control group |                           | 10% necrosis flaps control                   |
| Cunningh and Shons\(^{43}\) | Rat Fischer–Lewis | 20 | Neoadjuvant                            | Groin            | 4 Gy    | Arterial/venous anastomosis | Statistically significant differences 5% Arterial thrombosis |
|                      |                     |    | Fractionated dose                      |                  | 6 Gy    | Femoral                   | 31% venous thrombosis RT                    |
|                      |                     |    | 6wk pre SP                             |                  | Control group |                           |                                               |
| Krag et al\(^{42}\) | Rabbits New Zealand | 20 | Neoadjuvant                            | Cervical         | 35 Gy   | Arterial/venous anastomosis | Thrombosis RT 20%                           |
|                      |                     |    | Fractionated dose                      |                  | Control group |                           | Thrombosis no RT 0%                          |
|                      |                     |    | 6wk pre SP                             |                  |          | Arterial/venous anastomosis | Statistically significant differences 2% arterial thrombosis |
|                      |                     |    |                                      |                  |          | Carotid/jugular           |                                               |
| Ragnarson et al\(^{44}\) | Rabbits New Zealand | 20 | Neoadjuvant                            | Cervical         | 20 Gy   | Arterial/venous anastomosis | No statistically significant differences 0% venous thrombosis |
|                      |                     |    | Single dose                            |                  | Control group |                           | 2.5% arterial thrombosis                     |
|                      |                     |    | 4wk pre SP                             |                  |          | Carotid/facial            | No control group                            |
|                      |                     |    | 20wk pre SP                            |                  |          |                          |                                               |
| Aitasalo et al\(^{32}\) | Rat Wistar Albino | 13 | Neoadjuvant                            | Groin            | 20 Gy   | Epigastic free flap to femoral vessels | No statistically significant differences 23.1% Thrombosis RT |
|                      |                     |    | Single dose                            |                  | Control group |                           |                                               |
|                      |                     |    | 1wk pre SP                             |                  |          | Arterial anastomosis      | Thrombosis control 18.2%                     |
|                      |                     |    |                                      |                  |          | Femoral                   | Statistically significant differences         |
| Arinci et al\(^{41}\) | Rat Sprague–Dawley | 40 | Neoadjuvant                            | Groin            | 20 Gy   | Epigastic free flap to femoral vessels | Thrombosis RT group 13.3%                  |
|                      |                     |    | Single dose                            |                  | Control group |                           | Thrombosis control 0%                        |
|                      |                     |    | 2wk pre SP                             |                  |          | Arterial anastomosis      |                                               |
|                      |                     |    |                                      |                  |          | Femoral                   |                                               |
| Takan et al\(^{52}\) | Guinea pigs         | 18 | Neoadjuvant                            | Groin            | 20 Gy   | Epigastic free flap to femoral vessels | No statistically significant differences 19% Thrombosis RT group |
|                      |                     |    | Single dose                            |                  | Control group |                           |                                               |

RT, radiotherapy; SP, surgical procedure.

series were extremely small.\(^{6,34,42,44}\) To overcome this methodological mistake, we used the statistically recommended number of animals in each group providing an adequate statistical power. Previous experimental studies also show differences in the applied surgical technique,\(^{31,32,43,45}\) radiation dose, and animal models\(^{31,32,43,45}\) that impede a direct and objective comparison.\(^{31,32,43,45}\) There is only 1 previous study in rats evaluating the patency rates of anastomosis in artery and vein\(^{46}\) but it had significant methodological failures such as the small series used, insufficient radiotherapy dosage (2.5–9 Gy),\(^{41}\) and the long interval between the neoanastomosis and the moment of the surgery, as compared with the current clinical protocols.\(^{7,10–12,14,30}\)

In our experimental study, apart from providing enough statistical power, we tried to decrease confounding factors to isolate the relations between anastomotic patency, which is believed to be the most important factor in microvascular surgery,\(^{31,42,43,45,48,49}\) and radiotherapy. Thus, we did not opt for an irradiation model over a flap’s pedicle but a model of 2 independent vessels in the same surgical field approached by a single incision. After a flap necrosis due to a vascular alteration, it is not possible to discriminate the origin of the thrombosis in either artery or vein.\(^{31,32,43,45}\) In addition, we decreased other causes of flap failure such as infection and wound dehiscence, more frequent in free flaps.

The dosage used in our experimental model is greater than those administrated in other previous models, which used doses from 2.5 to 15 Gy, even using fractioned doses.\(^{27,28,41,42,45}\) We have selected this radiotherapy method with a radiotherapy of 20 Gy unit dose to reproduce with maximum fidelity all the adverse clinical conditions and to favor the appearance of postoperative radiotherapy local side effects. Post-radiotherapy arterial and venous histological changes, such as a reduced number of endothelial cells, reduction in the number of smooth muscle nuclei in the media, intimal proliferation, edema, and fibrosis of the adventitial tunica, were similar to the changes observed in tissue samples from clinical studies.\(^{27,28,36,41}\) Significant histological changes when comparing endothelial cells, intimal layer, and muscular layer between radiotherapy and non-radiotherapy groups were considered to be due to radiotherapy. Changes in external elastic lamina, medial layer, and adventitia were considered to be due to surgical procedure.

Although macroscopically normal-looking through the microscope, the irradiated venous showed a high rate of thrombosis (20%) at the end-to-end anastomotic site, compared with irradiated arterial (3%) and nonirradiated recipi-
ent (0%) vessels. Furthermore, higher surgical difficulties as reflected in the higher number of attempts and thus an increased surgical time and number of stitches have been found in the irradiated group. Microsurgical anastomoses are largely technically dependent; however, there exists a finite rate of failure, with often devastating consequences, such as venous thrombosis, the most frequent complication.56–58 We could not confirm that there were not mechanical traumas associated with the repeated number of attempts applied to the irradiated vessels. Repetitive attempts of vascular anastomosis are traumatic and should be performed as gently and infrequently as possible.50 According to our thrombosis results on non-radiotherapy groups (0/70), we could obviate the technical error as the cause of vein thrombosis.

Both arteries and veins showed histological changes related to radiation. However, only veins showed a significant increased ratio of thrombosis. Tissular changes related to radiotherapy, a thin-walled vessel32,41 (ie, vein), the technical difficulties in the execution of the microanastomosis, and the slower blood flow in the venous system might be the main factors contributing to thrombus formation.60 All the thrombosed vessels needed more than 1 attempt to obtain a permeable anastomosis. Thus, trauma related to repetitive attempts of anastomosis on a pathologic and thin-walled vessel could explain the increased rate of vessel thrombosis. We would support those authors recommending to perform a dual vein anastomosis whenever possible2,15,16,49 and the use of pharmacologic agents to reduce the rate of thrombosis after free tissue transfer.61

The current study, performed in an animal model, presents inherent limitations in the clinical translation of the information obtained. We chose an anastomosis model instead of a free flap model, which is very different from a clinical scenario, but it allows us to reduce confounding factors that might influence the viability of the anastomosis. Similar to other preclinical studies, other limitations of the model are the radiotherapy-induced changes at both ends of the anastomosis, different from patients where the tumor bed vessels are irradiated but the free flaps vessels are not irradiated.31,32,43,45 We believe that this research provides valuable contribution to current knowledge as our results demonstrate that irradiated vein anastomoses have higher failure rates than irradiated arterial and nonirradiated recipient vessels. However, further studies are still needed to determine the role of each theoretical factor contributing to the development of vein thrombosis so it will allow surgeons to design the strategies to prevent it.

CONCLUSION

Preoperative radiotherapy influences viability of the vein anastomosis in a rat model with a significant increase in the incidence of vein thrombosis.

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ACKNOWLEDGMENTS

We would like to thank Vall d’Hebron Research Institute, A. Rojo, and M. Rosal for their precious help in the care of the animals. We would also like to thank the Radiotherapy and Pathology departments of Hospital Universitari Vall d’Hebron for their practical suggestions and encouragement.

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