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The histopathologic evaluation of soft tissue changes in rabbit extremity after different dose-fractionation schemes of interstitial high dose rate (HDR) brachytherapy

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ABSTRACT

Background: The use of HDR in the treatment of soft tissue sarcoma had been on the rise. However, there was limited study to evaluate the effect of different fractionation schemes on soft tissue and the optimal HDR scheme.

Aims: We aimed to assess the histopathologic changes on soft tissue after different HDR brachytherapy doses.

Methods: The subjects were divided into three groups. Each group included 10 limbs. Group A had only an applicator without radiation, group B received a total of 24 Gy at 6 Gy per fraction, twice a day, and group C received a total of 13.5 Gy in a single fraction. The histopathologic findings were grouped into soft tissue pathology-1 (edema, inflammation, endothelial proliferation, necrosis) and soft tissue pathology-2 (atrophy, calcification, vascular hyalinization, fibrosis) (STP-1–2).

Results: The highest mean grade values of STP-1 and STP-2 were observed in group C (0.95 and 1.45) in comparison to group A (0.45 and 0.85) and group B (0.65 and 0.9). The difference in STP-1 was found significant only between groups A and C and the difference in STP-2 was found both between groups A and C and groups B and C.

Conclusion: In our experimental study it was shown that the fractionated interstitial HDR had both lower rate and severity of toxicity in comparison to a single high dose fraction. Before using a single fractionated regimen in the clinic, the increased morbidity related to the irreversible early toxicities or progressive late toxicities should be kept in mind.

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1. Introduction

In recent years, the tissue damage caused by irradiation has been explained by three mechanisms. First one is a well-known phenomena characterized by a direct injury with the mitotic death of parenchymal cells. The second one is currently accepted as the main mechanism and explains the early and late responses of losing the parenchymal and stromal cells after microvasculature injury.[1,2] Furthermore, there is a new paradigm that explains the third mechanism by the active biologic response to radiation in normal tissues with the release of cytokines, growth factors and chemokines.[2–4] Nevertheless, the time and dose of radiotherapy with a fractionation scheme are as important as molecular and cellular changes in normal tissue induced by radiation.[2] All these mechanisms are related with each other and affect the severity of early and late toxicity which limits the deliverable curative dose of radiotherapy (RT), and might affect the life quality of the patient.

Although many different dose and fractionation schedules have been improved until today, the conventional fractionation regimen (1.8–2 Gy/once a day) has been preferred frequently for curative treatment of the most solid tumors.[5,6] The reason being that better local control rates with fewer toxicity incidences are obtained by a conventional regimen in comparison to altered schemes. However, the application of the conventional regimen to the radioresistant tumors such as malign melanoma or soft tissue sarcoma may be a detrimental for treatment success. It was reported that better treatment results are achieved in hypofractionated scheme in curative treatment or single fraction scheme in palliative treatment of soft tissue.[5,7–10] Moreover, in recent years, it has been shown that higher local control rates in soft tissue sarcomas have been provided by intraoperative[11,12] or postoperative.[13–15] High dose rate (HDR) brachytherapy with hypofractionated schemes in single/multiple fractions. The acceptable toxicity for different dose-fraction schemes of HDR, such as 15 Gy in a single fraction or 36–50 Gy in multiple fractions, was reported.[13]

The lower rate of toxicity may be the result of the application of high dose radiation to the limited therapy areas rather than external RT. One of the major advantages of brachytherapy is that it can lead to dose optimization. In this process, the isodose volumes in the tissue can be created by a combination of careful placement of catheter and the adjustment of dwell times of a computerized stepping source.[16] However, the disadvantage is that the dose distribution is inhomogeneous since the dose fall off is very rapid. This can result in increased morbidity if hot spots occurring within critical normal tissues. The dose inhomogeneity can be minimized, but not eliminated, by optimizing the dwell times in HDR.[17] Therefore, it is not possible to estimate the severity of early and late reactions in adjacent normal tissue that are exposed to high dose radiation. Also, the high dose regions of the implant have a large number of normal cells which consequently may lead to severe normal tissue complications.[18] Additionally, the severity of side effects can be increased by different fractionated regimens of HDR.

In the light of radiobiologic knowledge, we know the effects of radiation on soft tissue. But there is no experimental study or prospective randomized clinical trial that evaluated the normal tissue damage of different dose-fractionation HDR which notably is the current treatment modality for soft tissue sarcoma. The trials in the literature are retrospective with limited number of patients and the heterogeneous therapies applied. This fact led us to evaluate the histopathologic changes on soft tissue of a rabbit extremity after different HDR doses both in a single and multiple fractionation schemes.

2. Materials and methods

The experimental protocol was approved by Gazi University School of Medicine Experimental Animals Ethic Committee by decision number of G.U.ET-05.038. The experiment was carried out in compliance with the 3R (reduction, replacement, refinement) ethical guidelines. During the study, the animals were being housed in the Animal Breeding and Research Laboratory of Gazi University School of Medicine, at a constant temperature ($22 \pm 1\,^{\circ}C$) and were bred with standard feed. The study was conducted at Gazi University, Department of Radiation Oncology.

The rabbits were preferred as subjects as suggested by the literature in which the soft tissue damage of radiation was assessed.[19–22] A total of 10 New Zealand rabbits (male, 1500 g on average, 2 months old) were used. It was planned to use three extremities of each animal. The same extremities were used for a particular treatment group in each animal. Each group included 10 extremities. It was planned that the first group (group A-right fore-limb) had only an applicator without radiation, the second group (group B-right hind-limb) received a total of 24 Gy at 6 Gy per fraction, twice a day, and the third group (group C-left hind-limb) received a total of 13.5 Gy in single fraction. To evaluate the variation in the effectiveness of different fractionation doses and to compare the two treatment regimens radiobiologically, the following formula was used:

$$Dr/Dx = \alpha/\beta + dx/\alpha/\beta + dr$$

[Dr: known total dose (reference dose), Dx: new total dose (with different fractionation schedule), dr: known fractionation (reference), dx: new fractionation schedule].[23]

The α/β ratio was accepted as 3.5 due to muscular, connective and vascular tissues involved in late reacting tissues.[24]

According to this formula, 13.5 Gy, 24 Gy, 13.5 Gy and 6 Gy were determined for Dr, Dx, dr and dx, consecutively. After calculation, it was shown that the effectiveness of 13.5 Gy in a single dose fraction was equal to 24 Gy in 6 Gy per fraction radiobiologically ($229.5 \approx 228$).

The animals were anesthetized with an intramuscular injection of xylazine HCl at a dose of 5 mg/kg and ketamine HCl at a dose of 50 mg/kg, prior to catheterization and brachytherapy.

For brachytherapy, a HDR afterloading system (Nucletron, Holland) was used. After anesthesia, under sterile conditions, through- and- through implant technique was used. The steel guide needle was inserted through the extremity. The leader (tapered end) of the flexible nylon catheter (5 French in diameter, 30 cm in length) was passed down the needle and both the needle and catheter were pulled through the other end.

After the needle was removed, the catheter was pulled until the closed end of it topped with a nylon button was in contact with the surface. Another nylon button was inserted at the opposite end of the catheter and was cut at 3 cm from the surface. The entry and exit sites of the catheter were marked with Indian ink.

After marking, a dummy wire was placed inside. Before planning, the simulation films of all extremities of animals were taken at 30 and 330 gantry degrees to determine the source localization. The second, third and fourth catheter points, which indicate the soft tissue, were selected as reference. In the treatment planning system, the planning (with x–y–z coordinates) were performed for the application of brachytherapy at a dose of 13.5 Gy for group C and 6 Gy for group B. It was planned to irradiate the same tissue volume giving the determined dose to 1 cm away from the source. After the dummy wire was removed, the Iridium 192 wire (dose rate was 15.69 Gy/h) was inserted into the catheter with after loading remote control system and the subjects were irradiated. For group B, at least 6-h interval between the fractions was allowed to let the sublethal damage repair on normal tissue.

Before sacrificing, the differences in skin and hair, and the presence of necrosis, and the function of extremity were evaluated once a week after brachytherapy. The animal was anesthetized and sacrificed by intramuscular xylazine HCl 20 mg/kg and ketamine HCl 200 mg/kg, at the end of the 4th week following brachytherapy. All the three extremities were removed from the rabbit and put into caps with 10% formalin. Each cap was numbered from 1 to 30 and sent to Gazi University Pathology Department.

For histologic examination, a thick tissue between the applicator entry and exit sites marked by a tattoo was removed from the extremity. Two transverse sections of thick tissue, including one from medial and lateral axis which are 1 cm away from the central axis (source axis) – which predicted the tissue that received the determined dose – were obtained and embedded in paraffin. Five-micron thick tissue sections were provided and stained with Hematoxylin–Eosin. Each section was assessed by a pathologist who was blinded to study the groups. He evaluated the specimens for edema, inflammation, endothelial proliferation, muscle degeneration, atrophy, calcification and fibrosis. The scoring system described by Baker and Leith[25] was used. According to the scoring system, five grades were used. The grading was determined by assessing the percentage of lesion in a slice. A detailed description of grades is shown in Table 1. After the determination of grading scores in two sections, the highest scoring value for each extremity was taken for statistical analysis.

Table 1 – Histopathologic grading system.

Grade 0	None	Normal appearance
Grade 0.5	−/+	Characteristic lesion is seen but cannot be differentiated definitely
Grade 1	+	Minimal lesion is apparent (≤25 of the slice)
Grade 2	++	Moderate degree lesion (>25 to ≤50 of the slice)
Grade 3	+++	Very severe reaction (>50 of the slice)

Statistical analyses were performed with SPSS (Statistical Package for Social Sciences) 13.0 version. The χ^2 and Fischer Exact tests were used to compare the qualitative data and Mann–Whitney *U* test was used to compare the mean values of quantitative data. The mean values were given with standard variations and minimum–maximum ranges. *p* values of <0.05 were considered to be significant.

3. Results

At physical examination, no neurologic deficit, loss of extremity function and death were observed. At the end of the 3th week, the hair loss on 6 (60%) extremities in group B and 7 (70%) extremities in group C were noted.

At histopathologic evaluation, a total of 30 extremities were assessed. The soft tissue pathologies (STP) were separated into two groups such as STP-1 including edema, inflammation, endothelial proliferation, necrosis; and STP-2 including atrophy, calcification, vascular hyalinization and fibrosis. However, as well as the acute reactions, the starting of histopathological findings such as atrophy, calcification, vascular hyalinization and fibrosis at the end of the 4th week after radiation, which was determined as latent period, led us to evaluate those as the beginning of late histopathologic reactions.

The frequencies of STP grades in study groups were analyzed according to four grading scores (Grades 0, 1, 2, 3) as no grade 0.5 STP was observed (Table 2). After that, the mean values of STP grades were analyzed. For STP-1, it was 0.45 ± 0.23 (minimum–maximum 0.25–1) in group A, 0.65 ± 0.65 (minimum–maximum 0–1.75) in group B and 0.95 ± 0.56 (minimum–maximum 0.25–1.75) in group C. In comparison of the mean values between study groups, the significant difference was found only between group A and C ($p = 0.041$). The differences between group A and B or group B and C were 0.617 and 0.222, respectively. For STP-2, the mean value was 0.85 ± 0.56 (minimum–maximum 0.25–1.5) in group A, 0.9 ± 0.21 (minimum–maximum 0.75–1.25) in group B and 1.45 ± 0.45 (minimum–maximum 0.75–2) in group C. In comparison of mean values between study groups, the significant difference was found both between groups A and C ($p = 0.021$)

Table 2 – The frequencies of STP grades in study groups.

	Study groups		
	Group A ($n = 10$)	Group B ($n = 10$)	Group C ($n = 10$)
STP-1			
Grade 0	–	4	–
Grade 1	9	2	6
Grade 2	1	2	2
Grade 3	–	2	2
STP-2			
Grade 0	–	–	–
Grade 1	6	4	2
Grade 2	4	6	4
Grade 3	–	–	4

STP: soft tissue pathologies; group A: control arm; group B: fractionated HDR arm; group C: single fractionated HDR arm.

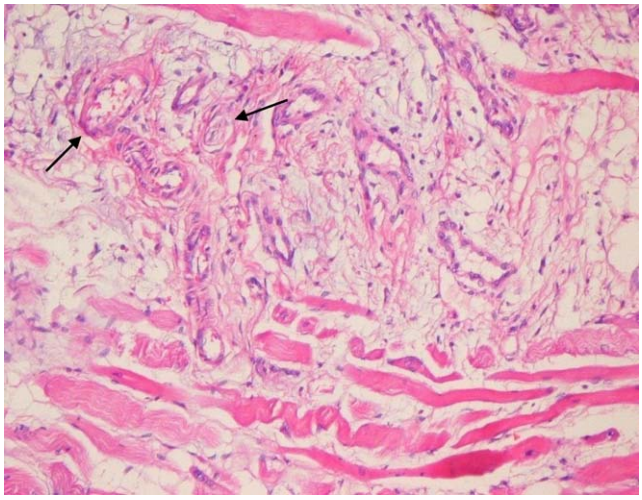

Fig. 1 – The irregular shape and diameter of vessels, HE×400.

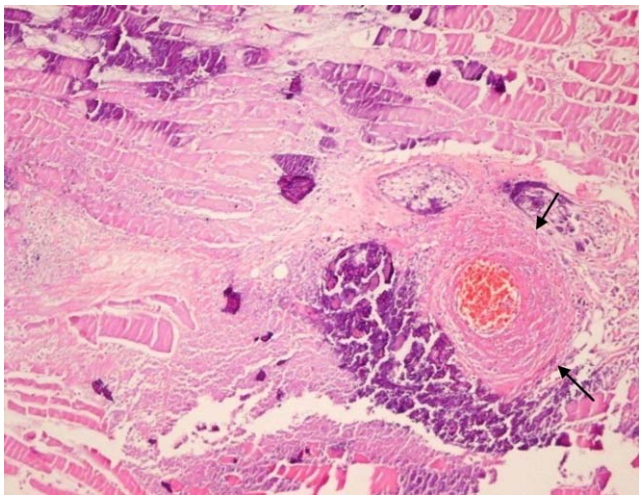

Fig. 2 – The fibrin accumulation, necrosis and calcification in vessel wall, HE×40.

and groups B and C ($p = 0.008$). The difference between groups A and B was not found significant.

After evaluation of STP-1 and STP-2 values with the quartet scoring system, two subgroups of STPs, low grade (grades 0, 1) and high grade (grades 2, 3), were constituted. In comparing the distribution of low and high grades of STP-1 between study arms, the endothelial proliferation ($p = 0.043$) between groups A and B; the edema ($p = 0.012$), the endothelial proliferation ($p = 0.005$), the necrosis ($p = 0.003$) between groups A and C; and the necrosis ($p = 0.035$) between groups B and C were found significant (Table 3). In Fig. 1, the irregular shape and diameter of vessels and endothelial proliferation, in Fig. 2 the necrosis

Table 3 – The distribution of low and high grade of soft tissue pathologies among study groups.

	Study groups								
	A (n =10)	B (n =10)	p	A (n =10)	C (n =10)	p	B (n =10)	C (n =10)	p
STP-1									
Edema									
LG	8	4	0.085	8	2	0.012*	4	2	0.314
HG	2	6		2	8		6	8	
Inflammation									
LG	2	6	0.085	2	2	0.709	4	2	0.085
HG	8	4		8	8		6	8	
Endothelial proliferation									
LG	10	6	0.043*	10	4	0.005*	6	4	0.371
HG	–	4		–	6		4	6	
Necrosis									
LG	9	7	0.291	9	2	0.003*	7	2	0.035*
HG	1	3		1	8		3	8	
STP-2									
Muscle atrophy and deformation									
LG	9	4	0.029*	9	–	0.000*	4	–	0.043*
HG	1	6		1	10		6	10	
Calcification									
LG	6	4	0.328	6	2	0.085	4	2	0.314
HG	4	6		4	8		6	8	
Vascular hyalinization									
LG	10	10	–	10	6	0.043*	10	6	0.043*
HG	–	–		–	4		–	4	
Fibrosis									
LG	7	7	0.686	7	2	0.035*	7	2	0.035*
HG	3	3		3	8		3	8	

STP: soft tissue pathologies; group A: control arm; group B: fractionated HDR arm; group C: single fractionated HDR arm; LG: low grade; HG: high grade.

* $p \leq 0.05$, as statistically significant.

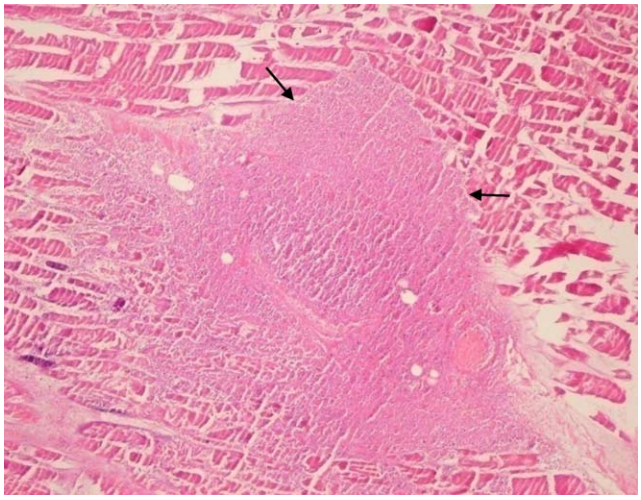

Fig. 3 – The necrosis in muscle tissue, HE×100.

in vessel wall and in Fig. 3 the necrosis in muscle was shown.

For STP-2, the differences of muscle atrophy and deformation ($p = 0.029$) between groups A and B; muscle atrophy and deformation ($p = 0.000$), vascular hyalinization ($p = 0.043$) and the fibrosis ($p = 0.035$) between groups A and C; muscle atrophy and deformation ($p = 0.043$), vascular hyalinization ($p = 0.043$) and fibrosis ($p = 0.035$) between groups B and C were found significant (Table 3). In Fig. 2, the calcification of vessel wall and in Fig. 4, the atrophy and fibrosis of muscle were shown.

4. Discussion

The soft tissue damage after external radiotherapy or brachytherapy, which is not frequently seen as well as mucositis or enteritis, cause serious effects. The incidence of soft tissue damage according to various treatment schemes is reported as 0–40%.[26] It is frequently seen after breast and head and neck cancer or soft tissue sarcomas. Although the high local control rates for soft tissue sarcomas after wide local excision and RT are reported,[27] the increased morbidity of wide area radiation after surgery limits the effectiveness of the therapy. For this reason, in recent years, most clinicians have preferred brachytherapy applied on a limited area without external RT.[13] However, there is no consensus for optimal dose and fractionation scheme of brachytherapy. In retrospective series, frequently preferred HDR schemes after external RT (45–50 Gy) were 15–25 Gy in a single or multiple fraction.[13,28] If HDR is used alone, 42–45 Gy at 4–6 fractions or 36 Gy in 10 fractions, twice a day, are recommended.[13] For pediatric patients, the recommended postoperative HDR dose without external radiation is 36 Gy (in 12 fractions, twice a day), which is lower than that for adults',[28,29] and the intraoperative HDR after external RT was applied at a dose of 12–15 Gy in a single fraction for this group.[29]

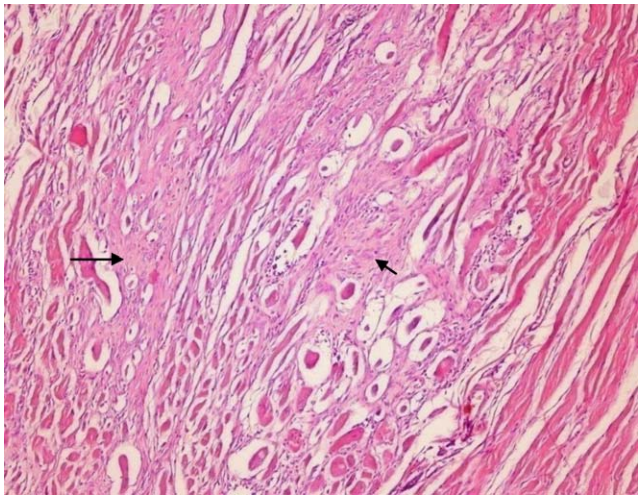

Fig. 4 – Atrophy and fibrosis of the muscle, HE×200.

In our study, the intended doses to evaluate were chosen considering frequently applied treatment schemes of 36 Gy in 12 fractions and single dose between 12 and 15 Gy to pediatric patients who are under higher risk of delayed side effects due to longer life expectancy. To assess the variation in the effectiveness of fractionation doses and to compare the treatment regimens radiobiologically, the formula which was mentioned before was used. According to the formula, when alpha beta ratio was accepted as 3.5 Gy, the radiobiological approximate value of 36 Gy in 12 fractions was 13.5 Gy in a single fraction ($234 \approx 229.5$). Since we think it would cause a heavier side effect due to larger fraction size, we wanted to compare a more hypofractionated regimen than that of 36 Gy with 3 Gy daily fraction dose to 13.5 Gy in a single fraction. Therefore, it was determined to apply 24 Gy with 6 Gy fraction size, in 4 fractions twice a day, after it had been shown to be nearly equal to that of 13.5 Gy ($228 \approx 229.5$).

Radiation effects on normal tissue are usually divided into two categories, namely early and late reactions. It is important to determine the difference between those for clinical presentation of treatment-related morbidity.[30] The morbidity that occurs within 90 days, typically 3–9 weeks after the start of RT has been defined as an early reaction; and the reaction that occurs after 90 days of radiation has been defined as late reaction; however, greater damage leads to a shorter latent period.[31] While cell loss and limited repopulating activity play major roles in the development of side effects, both acute and late, cascades of inflammatory and fibrogeneic cytokenes starting immediately after irradiation have also been identified. These events occur during the clinically silent, so-called latent period, and this period, before the late effects become apparent, is not biologically silent, as it is the time when cytokine cascades lead to the progressive tissue changes such as fibrosis.[32] Furthermore, it was shown in the study of Sener et al., that collagen contents of the studied tissues of rats after whole body irradiation were significantly increased 72 h following irradiation, despite the application of protective agent (Ginkgo biloba extract), indicating the presence of tissue fibrotic activity.[33] So, it is not a surprise to see late reactions such as fibrosis, histopathologically, at the end of the 4th week after brachytherapy as seen in our study. If the late reactions such as fibrosis are considered to have a tendency of being a progressive and irreversible process, it is certain that the ever-increasing evidence of fibrosis in histopathologic evaluation will become apparent in clinic in the following years. And it gives clinicians a message about

the rate and the severity of side effects which can increase consequently.

In general, the increased permeability in small blood vessels and the endothelial proliferation in large vessels are observed after first fraction of RT.[34] The increased permeability causes edema and inflammation in reaction to leukocytes' immigration to the damaged tissue and the release of cytokines.[35] As well as vascular changes, necrosis can also be seen after higher doses of above 10 Gy in an early period.[36] Therefore, in our study the histopathologic findings, such as edema, inflammation, endothelial proliferation and necrosis, were included in the STP-1 group which represents early reactions. After analyzing the distribution of the mean STP-1 grades in the groups, the highest values were observed in the single fraction HDR group (sHDR = group C) and the lowest values were observed in the control group (group A). In comparison of the mean values between study groups, the significant difference was only found between the control and sHDR groups. It can be explained by the fact that the application of a single fraction (single hit theory) in high dose causes a death of great number of parenchymal cells (the alpha component of linear quadratic model). If the radiation dose, especially the fraction dose, is increased, mitotic death occurs both in mature cells and limited proliferable soft tissue cells.[9,37] Since the remaining cells proliferate partially, the high ratio of parencymal cell loss cannot be compensated and the more intense acute reactions occur, such as the observed diffuse necrosis in the sHDR group. Also it supports the correlation between increased necrosis ratio and high doses. During the application of high doses in a single fraction, it should be remembered that the necrosis is irreversible and it may increase the severity of morbidity both in early and late period.

When the distributions of low and high grades of STP-1 between the groups were compared, the difference between control and fractionated HDR (fHDR) groups for endothelial proliferation and the difference between the control and sHDR groups for edema, endothelial proliferation and necrosis were found significant. No observation of evident toxicity in the fHDR group may be the reason for applying a total dose in fractions (multi-hit theory) and allowing a tissue repair between the fractions. Besides, the type and the kinetic of tissue play a major role in the development of early reactions rather than the fraction size and total dose.[9,37] The early effects of radiation are observed in rapidly proliferating tissues (skin, mucosa, intestinal epithelium, etc.) due to their high radiosensitivity in comparison to the slow proliferating tissues, such as the soft tissue, which show resistance to radiation.[5] No observation of significant difference in early reaction between control and fHDR groups supports this theory.

The tissue damage in early period is usually transient and frequently recovered. However, in tissue with low regeneration capacity, the acute inflammation can be healed with scar.[35] On the other hand, the vascular damage and cytokine release play a role in the development of scar and fibrosis. Many cytokines and growth factors are released by increasing inflammatory cells owing to increased vascular permeability. The chemokines activate the synthesis of extracellular matrix which starts the process of fibrosis.[2–4] Also, the radiation injury to the endothelial cells induces the proliferative activity. After a while, the generation of capillaries of irregular diameter and shape is seen (Fig. 1).[38] The thrombosis is observed owing to the narrow portion of these capillaries easily occluded by cells, and after that, the necrosis is observed at vessel walls (Fig. 2). The damage of the vessels impoverishes the nutritional quality of the extracellular fluid, ultimately leading to the loss of parenchymal cells and an increase in fibroblasts, and then fibrosis takes the place of dead cells (Figure 4).[34,39] Moreover as a result of tissue damage, the calcification can be seen in both vascular structures and parenchyma (Fig. 2). Since these findings are observed at the late period after radiation[30,35,40] and the processes in the development phases get along with our histopathologic findings, the atrophy, calcification, vascular hyalinization (collagen accumulation) and fibrosis were evaluated separately. In the assessment of STP-2 grades in the groups, the findings were more severe in the group of sHDR. It is known that the early reactions are related to daily and weekly radiation dose rather than the fraction size. However, the most important parameters for the late reactions are fraction size and the time interval between fractions.[5,9,41] In our study, while no difference was found in comparison of the mean values of STP-1 grades among the irradiated groups, the significant difference was found in comparison of STP-2 grades. This result shows the relation between the late effects and fraction dose. Also the severity of reactions in the fHDR group is lesser than that of the sHDR group and no evident difference between the fHDR and control group supports the necessity for the application of total doses in fractions and allowing at least 6-h interval for sublethal damage repair. To conclude, while lowering the fraction dose and allowing a definite time interval decrease the severity of late reactions, it has no major effect on early reactions.

5. Conclusion

Radiation related toxicity might negatively affect the tissue and organ functions with long-term health-related life quality of the patient. The hazard of toxicities may be higher than expected by the application of high doses of brachytherapy in soft tissue sarcoma. Our study aimed to evaluate the effects of different doses of interstitial HDR brachytherapy on soft tissue histopathologically. Our findings verified that increased rates and grades of side effects were observed in sHDR treatment. Therefore, if the aim is to cure the patient, high doses should be applied in multiple fractions with sufficient time interval. Otherwise, applying high doses in a single fraction regimen, which has a high toxicity rate in late period in contrast to early period, should be preferred for patients with less life expectancy. Before using this regimen as curative therapy to the patients, especially to pediatrics, the increased morbidity related to the irreversible toxicities such as necrosis or fibrosis should be kept in mind.

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