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# Posttreatment Histology and Microcirculation Status of Osteogenic Sarcoma after a Neoadjuvant Chemo- and Radiotherapy in Combination with Local Electromagnetic Hyperthermia

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**Key Words**
Osteosarcoma ■ Chemotherapy ■ Radiotherapy • Local hyperthermia • **Posttreatment histology** • Microcirculation

**Schlüsselwörter**
Osteosarkom • Chemotherapie ■ Bestrahlung • Lokale Hyperthermie • Histologie • Mikrozirkulation

## Summary

***Background:*** Many biological attributes of tumors (including regional blood flow and microcirculation) can deteriorate the homogeneity of heat distribution and temperature elevation during hyperthermia. We analyzed the connection between the microcirculation status of osteogenic sarcomas and the posttreatment histology after neoadjuvant chemotherapy, irradiation and local hyperthermia. ***Patients and Methods:*** 62 patients with histologically verified osteosarcoma (35 men, 27 women, age 9-53, average 21 years) were enrolled in the retrospective pathohistological study. 61 patients were evaluable. In 72.6% of cases the tumor was localized in bones forming knee joints. All patients received neoadjuvant treatment [6 hyperthermias (60 min, 42-45 ℃), daunorubicin 30-50 mg/m$^2$, 6 infusions, adriamycin or cisplatin 30 mg/m$^2$ for 3 days or once 90 mg/m$^2$ monochemotherapy before the hyperthermie procedure; subsequently 7-therapy, 20-36 GyJ followed by surgery; From archives, a control group was formed of 20 therapy-naive tumors. Resected tumors were histologically examined for assessment of spontaneous and therapeutically induced alterations. For analysis of the functionality status of microcirculation on histological cuts, 40 tumors (without selection) were investigated: 10 controls and 10 cases each with minimal, subtotal and total posttreatment alterations. ***Results:*** Chemotherapy and radiotherapy in combination with local hyperthermia induced a distinct damage to osteosarcoma. In 39.3 and 35.7% of cases there was subtotal and total devitalization of tumor parenchyma, respectively. Thrombosis of magistral and middle vessels, stasis in the microcirculation tree (collapse), damage to intimal vessels and endothelial cells, and necrotic alterations of the vessel walls appeared predominantly in central areas of tumors. Tumors with minimal devitalization of the parenchyma had a share of nonfunctional vessels ranging from 10.6 to 61.7%, mean 29.7%. In tumors with subtotal necrosis, between 34.5 and 72.0% (mean 49.46%) of vessels were nonfunctional (stasis, thrombosis). In 10 cases with 100% necrosis of the osteosarcoma parenchyma, a mean of 56.05% of nonfunctional yessels was registered (12.3-83.0%). In the control group, between 2.85 and 73!4% (mean 21.69%) of vessels showed damage to the microcirculation. *Conclusion :*There is a direct correlation between deterioration of the microcirculation in osteosarcoma and thermoradiochemotherapy-induced tissue alteration; the devitalization grade is directly proportional to the number of nonfunctional vessels in **the** tumor.

## Zusammenfassung

***Hintergrund:*** Regionaler Blutfluss, Mikrozirkulation und zahlreiche biologische Tumorattribute können die Homogenität der Hitzeverteilung und Temperaturexpansion wahrend einer Hyperthermie beeinflussen. Wir versuchten, den Zusammenhang zwischen Mikrozirkulationsstatus eines Osteosarkoms und Devitalisation des Tumorgewebes nach komplexer neoadjuvanter Therapie (Chemotherapie, Bestrahlung und lokaler Hyperthermie) zu bestimmen. ***Patienten und Methoden:*** In die retrospektive Studie aufgenommen wurden 62 Patienten (35 männliche, 27 weibliche) mit einem verifizierten Osteosarkom, Durchschnittsalter21 (9-53) Jahre. 61 Patienten waren evaluierbar. In 72,6% der Falle befand sich der Tumor in den Knochen des Kniegelenkes. Alle Patienten erhielten eine neoadjuvante Therapie [6 lokale Hyperthermien (60 min, 42-45 ℃), Mono chemotherapie i.v. oder i.a. mit Daunorubicin (DNR), 30-50 mg/m$^2$, 6 Infusionen, Adriamycin (ADR) oder Cisplatin (DDP), 30 mg/m$^2$ 3Tage lang oder einmalig 90 mg/m$^2$ vor der Hyperthermieprozedur; danach 7-Bestrahlung 20-36 Gy], gefolgt von einer Operation. Archivkontrolle:20Tumoren nach Operation ohne konservative Therapie. Jeder Tumor wurde histologisch zur Einschatzung spontaner und therapeutisch indizierter Veränderungen (Devitalisationsgrad) untersucht. Wir bewerteten die Funktionsfähigkeit der Mikrozirkulation in den histologischen Schnitten, wobei wir willkürlich 10 Falle aus jedem Devitalisationsgrad auswählten. ***Ergebnisse:*** Eine relativ niedrige Dosis Radiotherapie (20-36 Gy) und eine Monochemotherapie mit DNR, ADR oder DDP unter Anwendung von 5-6 Sitzungen lokaler Hyperthermie induzierten eine eindeutige Schädigung des Tumorgewebes: in 39,3% der Falle eine subtotale und in 35,7% eine totale Devitalisation des Tumorparenchyms. In den zentralen Bereichen der Tumoren beobachtete man verstärkt Thrombosen magistraler und mittelgroßer Blutgefäße, Storungen des Blutflusses und Blutstau im Mikrozirkulationssystem (Kollaps), Schädigung der Gefäßinnenwände und der endothelialen Zellen sowie abgestorbene Teile von Gefäßinnenwand. Periphere Tumorzonen zeigten zusätzlich deutliche Anzeichen einer Mikrozirkulationsstörung. Tumoren mit einer minimalen Devitalisation erreichten einen Anteil an nichtfunktionalen Gefäßen von 10,6 bis 61,7%, im Durchschnitt 29,7%. 34,5-72,0% - durchschnittlich 49,46% - der Gefäße in den Tumoren mit subtotaler Devitalisation waren nichtfunktional (Blutstau, Thrombose). In 10 Fallen mit einer totalen Parenchymnekrose registrierten wir durchschnittlich 56,05% nicht-funktionale Tumorgefäße. Die Untersuchung der Tumorgefäße in der Kontrollgruppe ergab 2,85-73,4% einer Mikrozirkulationsschadigung, im Durchschnitt 21,69%. *Schlussfolgerungen:* Es existiert eine direkte Korrelation zwischen der Verschlechterung der Mikrozirkulation im Tumorgewebe und seiner Alteration durch die Thermoradiochemotherapie; der Grad der Devitalisation ist direkt proportional zur Anzahl der nichtfunktionalen Gefäße im Osteosarkom.

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## Introduction

The blood flow in tumor tissue is often impaired and shows many variations in tumor relative to normal tissues. This (in connection with oxygen and nutrient supply, tissue pH distribution) can distinctly influence the response of malignant tumors to conventional treatment techniques [1]. Attempts to modify the microcirculation with *hyperthermia* (HT) and therefore the microenvironment and metabolism of tumor cells provide the oncologist with an important site for intervention (also taking into consideration the properties of HT alone to destroy tumor cells, radio- and chemosensitization, and the possibility of numerous HT effects that play a positive role in overcoming multidrug resistance). Microcirculation/drug interaction under hyperthermic conditions leads to an elevation of chemotherapy efficacy, with an increase in membrane fluidity and permeability, disorganization of membrane lipids, rise in penetration of intracellular cytostatics, membrane and cytoplasmic MDR protein denaturation, and inhibition of DNA repair, etc. [1-3].

Temperature expansion is directly dependent on regional blood flow and varying tissue thermal tolerance characteristics. Highly perfused regions might be under-heated and cause regrowth of tumor [4]. But during and after HT the blood flow and especially vessel structures do not stay intact. HT may lead to a further decrease in predominant pathological blood flow in the tumor with subsequent vessel destruction, disturbance of the microcirculation, and augmented heat sensitivity [2, 5, 6]. With a repetition of HT treatment it is possible to potentiate a chain reaction in microcirculation deterioration, which could be an effective agent for tumor devitalization. Studies concerning the morphological changes (devitalization or posttreatment histology) of osteosarcomas after HT in combination with either chemotherapy or radiotherapy are limited practically. We took an interest in the status of microcirculation of human osteosarcomas after HT, and investigated the post-therapeutic changes.and the morphology of the microcirculation tree of human osteosarcomas before and after a complex neoadjuvant therapy (chemo- and radiotherapy in combination with local electromagnetic HT).

## Patients and Methods

*Patients*

62 patients with locally advanced osteosarcoma (35 men, 27 women, aged 9-53, mean 21 years) were treated in conformance with Protocol Phase II Study in 1985-1990 in the Belarussian Scientific Institute for Oncology in Minsk, and were chosen for this retrospective pathohistological study. In 72.6% of the cases the tumor was localized in bones forming the knee joints. The pathologic diagnosis was established with a needle biopsy. 62 patients received a multimodal neoadjuvant treatment (chemotherapy, local HT, irradiation) followed by surgery. 20 patients (historical control group) underwent primary surgery.

*Histology*

28 tumors were determined as an osteolytic variant with well-defined cell polymorphism, grade 3 (poorly differentiated) tumors; 13 as an osteoplastic variant with predominantly neoplastic osteogenesis, grade 2 (moderately differentiated) tumors; in 41 cases we observed mixed tumors with features of osteolytic and osteoplastic variants, grade 3 (poorly differentiated).

*Treatment*

HT was administered twice per week. The total applications of regional HT ranged between 2 and 13 (average 6). 26 patients received local electromagnetic highest-frequency HT (460 and 915 MHz), 33 patients ultrahigh-frequency HT (40.68 MHz), and 3 patients received high-frequency HT (13.56 MHz). Each application lasted 60 min; a temperature of between 42 and 45 °C was attained in the tumor. The temperature in the tumor was controlled by an invasive method: elastic thermosonde with a semiconductor element were inserted in needles with 0.8 mm diameter or in plastic catheters with 1.2 mm diameter. Needles with thermosonde were put into the tumor immediately before HT; catheters were put into the tumor during diagnostic biopsy. All manipulations were done under short general anesthesia. To prevent burning of healthy tissue over the tumor, a water-cooling device was applied to the surface area. We combined HT and monochemotherapy with adriamycin (ADR), cisplatin (DDP) or daunorubicin (DNR) intravenously or intraarterially (in cases of tumor-involved extremities) for achieving a higher therapeutic ratio. Catheterization in the magistral arteria of the extremity was done with a Seldinger needle. ADR or DDP was administered in a single dose of 30 mg/m$^2$ for 3 days or once 90 mg/nr, DNR 30-50 mg/m$^2$,6 infusions before HT. The whole treatment. - regime was combined with distant 7-therapy, with an average regional dose of 20-90 Gy (in most of the cases 36 Gy was administered). Irradiation delivered in *2*- to 9-Gy fractions once per day was given to the tumor area from 3 fields located in an obtuse angle of 120°, 2-5 times per week. (The patients got radiotherapy right away after each HT.) After this treatment 23 patients received a tumor resection; in 39 cases an amputation was made. Surgery followed within a range of 2 days to 2 years. 36 patients were operated upon within the 1st month after therapy. One patient repudiated surgery after neoadjuvant therapy; surgery was carried out in 2 years, the tumor was not included for examination of microcirculation status.

*Morphological Study*

The efficacy of complex therapy was evaluated histologically. 7-18 formalin-fixed fragments of each tumor were cut out after decalcification for parafin embedding. Each fragment was sliced 8-12 times and colored according to conventional methods (hematoxylin-eosin). Special histochemistry methods were used for the identification of collagen, reticulin and elastin fibers (with pycrofuxin, asan, argentum nitricum, phuxelyn). For the assessment of histopathological response (HR) in tumors we used the Aymard method [8]. In a resected tumor after neoadjuvant therapy more than 5% of the viable tumor cells complied with the 2nd grade of HR, minimal devitalization. Less than 5% of the viable tumor cells corresponded with the 3rd grade of HR. A total devitalization of the tumors (100% necrosis of tumor parenchyma) matched with grade 4 of HR. Without any primary selection we analyzed the status of the microcirculation tree in 10 control tumors, in 10 tumors with HR grade 2, in 10 tumors with HR grade 3, and in 10 tumors with HR grade 4 after neoadjuvant therapy. We chose only cases which were operated upon during the 1st month after this therapy. Employing a light microscope linked with the semiautomatic picture analysis PC system 'Integral 2 MT' we measured on histological cuts with 17-fold microscope enlargement the total surface (um$^2$) of 50 vessels in every tumor. In all cuts from the central and peripheral areas of the resected tumor we gave first preference by measurement to vessels with direct symptoms of microcirculation disturbance (stasis, thrombosis). We defined such vessels as nonfunctional. Taking into consideration all nonfunctional vessel structures in all cuts, subsequently we would have measured the functional vessels (the lumen of the vessel was transparent), assuming with adequate surface an approximate total number of 50 vessels.

## Results

*Osteogenic Sarcoma before the Treatment* The morphology of osteosarcomas consists of predominant extensive vascular channels with chaotic architectonic; spontaneous destructive changes - necrosis, massive bleeding, and

Table 1. The microcircuiation status of osteosarcoma after thermoradio-chemotherapy'

| Devitalization Grade | Total surface of 50 vessels µm2 | Surface of nonfunctional vessels µm2 | % |
|---|---|---|---|
| * | 371,812 ± 56,747 | 97,526 ± 34,341 | 21.7 ± 6.7 |
| 2 | 676.522 ± 99,330 | 230,921 ± 70.572 | 29.7 ± 5.0 |
| 3 | 790,145 ± 100,850 | 418,252 ± 86,916 | 49.5 ± 4.2 |
| 4 | 659,781 ± 135,890 | 432,972 ± 124,398 | 56.0 ± 6.4 |

' Values are mean ± divergence ($p<0.05$). * Control group.

absence of elastin fibers - are typical in these vessels. Well-defined necrotic areas appear predominantly in an osteolytic variant of tumor. These reach up to 90% of the tumor volume, on average 22.5%. In osteoplastic tumors destructive changes were minimally represented (from 5 to 15%). In 10 tumors without conservative therapeutic intervention, between 2.85 and 73.4% of the investigated vessels showed damage of the microcircuiation, on average 21.69%. In 6 tumors the portion of nonfunctional vessels was less than 20%. In 1 case (osteolytic variant) nonfunctional vessels had prevailed (73.4%), with clearly perceptible spontaneous necrotic alteration (90%) of the tumor mass.

### Osteogenic Sarcoma after the Complex Treatment

A relatively low amount of regional-dose radiotherapy (20-36 Gy) and chemotherapy with ADR, DDP or DNR using 5-6 applications of HT (42-45 °C, 60 min) is associated with clear-cut damage of tumor tissue: in 39.3 and 35.7% of cases there was subtotal and total devitalization of tumor parenchyma, respectively. Damage of the tumor showed up promptly after using the first complex procedures in combination with HT>42°C and reached its maximum at the end of the treatment cycle*. The consequence of this therapy was an extended necrosis of the tumor parenchyma with predominate localization in the center of the tumors and distinct infirmities of vascular component of the tumor stromas. We detected no correlation between such attributes of a tumor as size; character of growth, and histological type, differentiated grade and thermoradiochemotherapy-induced HR grade. Thrombosis of magistral and middle vessels, obstruction of blood flow and stasis in the microcircuiation tree (collapse), damage of intimal vessels and of endothelial cells, and necrotic alterations of the vessel walls appeared predominantly in the central areas of the tumors. Likewise, peripheral tumor zones exhibited noticeable signs of microcircuiation disturbance. In tumors with the 2nd HR grade the share of nonfunctional vessels amounted to between 10.6 and 61.7%, mean 29.7% (230,921 ±70,572 urn$^2$, $p<0.05$). Between 34.5 and 72.0% of the vessels in tumors with the 3rd HR grade were nonfunctional (mean 49.46%, 418,252 ±86,916 ujn$^2$, $p<0.05$). Severe deterioration of the microcircuiation in osteosarcoma was obtained in tumors in which we found no viable parenchyma elements. Out of 10 cases with HR grade 4, we found up to 40% nonfunctional vessels in 6 tumors; in 3 cases this number achieved over 70%. In one examination of this group only 12.3% of the vessels appeared nonfunctional, but the tumor parenchyma was totally destroyed. The mean of nonfunctional vessels in tumors with HR grade 4 was 56.05% (432,972 ± 124,398 n-m$^2$, $p < 0.05$). There was a direct correlation between the deterioration of microcircuiation in the tumor and its alteration throughout the therapy. The HR grade was directly proportional to the number of nonfunctional vessels in the •"osteosarcoma (table 1).

### Discussion

The main factor in spontaneous alteration in tumors is a rela-.tive insufficiency of vascularization, which continuously increases with the growth of the tumor [9]. In osteosarcoma before the treatment, we observed well-defined heterogeneity of spontaneous microcircuiation infirmity: between 2.85 and 73.4% of the investigated vessels displayed disturbance of their function (stasis, thrombosis). The tumor has primarily predominant wide channels with chaotic architectonic, without elastin filaments which do not allow an active constriction of the vessels. This leads to the rapid slowing down of the microcirculation, to rising hypoxia in the tissue, and decreases the trophic of the tumor cells [10,11]. We obtained spontaneous devitalization of up to 90% of tumor tissue, showing a direct correlation with the number of nonfunctional tumor vessels - 73.4%. Significant variations in microcircuiation, oxygen and nutrient supply, tissue pH distribution, acute or chronic acidosis, and in the difference of bioenergetic status of cells are likely to be typical between different locations within a tumor. The tumors have also different histological types and grades of differentiation. These can have a decisive influence on the therapeutic response of malignant tumors to conventional irradiation, chemotherapy, HT, and the cell biology within tumors [1,7,12]. HT is the strongest modifier and sensitizer to chemotherapy and radiation therapy. HT alone can induce massive destructive changes in the tumor. Tumor blood perfusion is the major factor counteracting the temperature rise in malignant tissue during HT [13-18]. On the other hand, we observed in one osteosarcoma (with only 12.3% of nonfunctional vessels) a total devitalization of the tumor parenchyma, which substantiates a clear-cut direct antitumor damage of the thermoradiochemotherapy. Heating at high temperatures (44-46 °C) causes vascular damage and reduces the blood flow, while mild-temperature HT, 40-43 °C, increases the blood flow in the tumor with little vascular damage [19]. In vivo investigations demonstrated that the mild HT with 42.5-43 °C intensifies the blood flow and tumor tissue oxygenation [19-22]. This raises the effectiveness of the radiation and supports the transport of cytostatics in the tumor. Waterman et al. [23] observed a tendency for blood flow to increase following the initial heat fraction at points where the steady-state temperature was approximately 41 °C or less. The presence of hypoxic cells in tumors is believed to be a major factor in limiting the effectiveness of radiotherapy and certain chemotherapy [24]. As a

result of the mild HT there is an efficacious cytostatic concentration in previously circulatorily disordered areas of the tumor [25] and a rise in oxygenation.

Anomalous tumor blood flow provokes the non-even subdivision of oxygen and nutrients within the tumor mass. Alterations of the vascular tree and substrate depletion are highest, presumably one of the leading causes of the apoptosis of tumor cells (in connection with directly destructive effects of HT). This hypothesis was tested and validated in an experiment with tumor cells grafted after HT [12]. When the transplantation was made immediately after HT, 27 of 28 animals developed a tumor; 24 h after HT the growth of the tumor was registered in • 2 of 20 animals. In these cases, the tumor developed from fragments with a temperature of 42 °C throughout the HT. These results show a correlation with other inquiries [13-17]. We observed damage of the blood capillaries in the tumor stroma after HT [2], and registered ultrastructure alterations in their endothelium cells, defects of capillary walls, hemorrhage and thrombosis. Capillaries were the most sensitive structures to HT in lung tumors [26]. Alteration of the vessel walls and microcirculation obstruction potentiated the direct thermic tumor cell damage and reduced the quantity of vital cells [15]. Subsequent HT applications could amplify the inhibition of protein synthesis and DNA repair [1-3]. The following factors lead to the inhibition of microcirculation in the tumor by the use of local HT: swelling of the endothelium cells comply with caryopyknosis and caryorexis (apoptosis). damage and rupture of the capillary walls and hemorrhage, adhesion of leukocytes and erythrocytes to the vessel walls, raising rigidity of the erythrocyte membrane and raising blood viscosity, and stimulation of the production of intercellular adhesion molecule-1 [17,27].

In conclusion, blocking of the microcirculation in tumors brings on oxygen deficiency, hypoxia, substrate depletion, heat congestion, increase in lactic acid concentration, decrease in pH, activation of lysosomal enzymes, inhibition of DNA repair, and disturbance of normal cell functions. These factors can ' induce secondary necrosis in anoxic tumor areas and enhance the process of apoptosis. The damage to the tumor appears promptly after using the first complex procedures (radiochemotherapy) in combination with HT>42°C and reaches the maximum at the end of the treatment cycle [28]. Considerably later, 5-6 weeks after radiotherapy, degenerative alterations of the tumor could be registered [29,30].The repetition of the complex therapy supports the chain reaction of damage of the tumor [31,32].

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