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ORIGINAL ARTICLE

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Neuromuscular damage after hyperthermic isolated limb perfusion in patients with melanoma or sarcoma treated with chemotherapeutic agents

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Abstract *Purpose*: To evaluate the incidence and entity of muscle damage after hyperthermic limb perfusion (HLP) with doxorubicin or melphalan, two widely used chemotherapeutic agents. *Methods*: We collected muscle biopsies from eleven patients with lower limb sarcoma or melanoma immediately before and at a variable time after the chemotherapeutic procedure (mean = 49.4 days). Biopsy specimens were stained with standard histochemical and immunohistochemical methods on cryostat sections and the grade of fiber atrophy was calculated. *Results*: Clear neurogenic alterations were present in pre-HLP biopsies of seven patients related to age and previous therapy. In six patients, the comparison between biopsies before and after HLP demonstrated worsening of preexisting neurogenic condition and appearance of mitochondrial-related damage. Reduction in type I or type II fiber diameter was present in nine patients, but no relation to doxorubicin or melphalan treatment was clear. An unexpected, large accumulation of desmin was detected in the muscle biopsy of one patient receiving doxorubicin, probably related to the mechanism of doxorubicin-induced myotoxicity. *Conclusions*: The observed neuromuscular toxic effects could be related to the physical or chemical conditions of HLP, in particular perfusion temperature; in addition, the present study demonstrates that preexisting neuromuscular changes, i.e. neuropathy, modulates the degree of further damage following HLP.

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Introduction

Hyperthermic isolated limb perfusion (HLP) with chemotherapeutic agents is an effective therapy for isolated limb tumors. The local delivery of chemotherapeutic agents prevents systemic toxicity and allows a higher concentration in the tissues affected by tumor. Various chemotherapeutic agents have been used, mainly on the basis of tumor type (i.e. melanoma, sarcoma). Melphalan is usually employed for the treatment of melanoma, while doxorubicin is used in different tumors including sarcomas, lymphomas, and breast cancer. Neurotoxicity is one of the most troublesome problems with HLP; its reported incidence varies from 1 to 48% [25]. It has been suggested that the damage to the neuromuscular system is due to many factors, such as flow rate, total melphalan dosage, or peak melphalan concentration, while the most important factor seems to be the perfusion temperature. In addition, the preexisting condition of the patient's muscle can influence the development and outcome of neuromuscular damage. However, until now, no data have been collected to support this point.

We have studied muscle biopsies before and after the HLP procedure and compared our data with the clinical outcome and technique parameters. The accumulation of desmin observed in the muscle biopsy of one patient from our series provides a clue to the mechanism of doxorubicin myotoxicity. It is well known that the utilization of doxorubicin is limited by a severe cardiomyotoxicity that can cause irreversible congestive heart failure.

Materials and methods

Patients

From a large series of patients undergoing hyperthermic limb perfusions with doxorubicin or melphalan in our hospital (about

50 patients from 1996) we selected eleven patients in which it was possible to perform a second muscular biopsy during a second intervention for complete resection. A first biopsy was performed before the chemotherapeutic procedure, and a second muscle biopsy was carried out at a variable time, in the occurrence of surgery for resection of tumors, all located in lower limbs.

We usually performed muscle biopsy in patients undergoing HLP immediately before they started the chemotherapeutic perfusion but, in addition, the patients enrolled in our study had a second biopsy performed during the second intervention, after an average of 49.4 days (range 15–105). Clinical and neurological assessments were performed before the HPL and at the time of the second biopsy.

The mean age of the patients was 58 years (range 20–76); their characteristics are listed in Table 1. After the procedure the patients were evaluated for acute HLP side effects, according to the Wieberdink scale [27], and for serum electrolytes, myoglobin, CK, and other blood parameters to detect fluid and electrolyte disturbances or the appearance of compartmental syndrome and rhabdomyolysis.

Hyperthermic limb perfusion

Drugs were injected directly into the oxygenator of the extracorporeal circuit when the entire lower limb had reached a homogenous temperature considered adequate to maintain a steady state of temperature. Minimum and maximum limb temperatures were monitored by multiple thermocouple probes inserted into the subcutaneous, muscular and neoplastic tissue. A temperature range between 41.5 and 41.8 °C was considered optimal and, therefore, selectively employed.

We administered doxorubicin at a dose of 1.4 mg/kg body weight (b.w.) to five patients affected with lower limb sarcoma, and melphalan at a dose of 10 mg/l of limb volume in six patients affected with lower limb melanoma.

To evaluate the neurotoxicity we compared the two muscle biopsies, performed before and after the HLP procedure, in the occurrence of resection of the primary tumor.

Muscle biopsy

Muscle biopsies were performed at the gastrocnemius level in most of the patients and were stained with standard methods on cryostat sections. The biopsied muscle specimens were immediately frozen in isopentane cooled with liquid nitrogen and then cut. Frozen sections (8 μm) were picked up on aminosilane-coated slides.

To evaluate the grade of atrophy and the occurrence of selective fiber type damage, the diameters of type I and II fibers were measured, counting about 100 fibers for each type in all the specimens, as described by Dubowitz [7]. Data concerning the diameters of type I and II fibers before and after surgery were analyzed with Student's *t*-test.

Immunohistochemical analysis was performed using monoclonal antibodies to desmin in all biopsies of all five doxorubicin-treated patients. Immunohistochemical analysis using monoclonal antibodies to titin, α-actinin, and spectrin was performed in the muscle biopsies of the patient with desmin aggregates.

Results

After HLP, all patients experienced a reversible grade II reaction according to the Wieberdink scale, transient pain being the most important symptom. No neurological sign developed on following clinical examination. These reactions are comparable with those described in the literature [25]. In fact, mild acute tissue reaction has been described in the majority of patients while long-term neuropathy has been reported in only 2% of patients after iliac perfusion and in none after femoropopliteal perfusion (2 and 9 of our patients, respectively).

In all patients CK slightly increased, ranging between 200 and 500 UI/l within 2 or 3 days after HLP. Only patient no. 7 showed an important increase in CK concentration, up to a peak value of 12.000 UI/l during the first week after HLP. The histological picture of this patient is described below. According to the patterns identified by Lai et al. [15], a CK value below 1000 UI/l between the first day and the first week after HLP is defined "early peak" and is not followed by severe limb toxicity. The morphological characteristics of the biopsies performed before and after HLP are shown in detail in Table 2. In one patient of our series, the pre-HLP biopsy resulted normal, whereas all other patients displayed some degree of alteration even in the muscle biopsy collected before the hyperthermic perfusion. Neurogenic changes were slight to moderate and were represented by target and target-like fibers, different degrees of type-grouping, type II fiber atrophy, and minor myopathic or mitocondrial alterations; often more than one of these features were found in the same patient.

Many factors might contribute to the subclinical signs of neuropathy observed in the pre-HLP biopsies of most patients, such as the occurrence of lumbosacral radiculopathy, particularly frequent in this age group,

Table 1 Characteristics of patients and previous treatment. *CT* chemotherapy, *RT* radiotherapy, *IFN* interferon

Patient	Sex	Age (years)	Tumor type	Level	Drug	Other therapies pre-HLP
1	M	44	Sarcoma	Left thigh	Doxorubicin	CT and RT
2	F	61	Melanoma	Right leg	Melphalan	CT and RT
3	M	55	Melanoma	Right knee	Melphalan	CT and IFN
4	F	42	Melanoma	Right thigh	Melphalan	RT
5	M	55	Sarcoma	Right leg	Doxorubicin	–
6	F	71	Melanoma	Left calf	Melphalan	IFN
7	M	70	Sarcoma	Left leg	Doxorubicin	–
8	M	20	Sarcoma	Right leg	Doxorubicin	–
9	F	68	Sarcoma	Right foot	Doxorubicin	–
10	F	75	Melanoma	Left foot	Melphalan	–
11	M	76	Melanoma	Right leg	Melphalan	–

Table 2 Histological characteristics of muscle biopsies before and after HLP

Patient	Pre-HLP	Interval (days)	Post-HLP
1	Change in motor unit and type II fiber atrophy	30	Worse
2	Some atrophic and angulated fibers as change in motor unit	38	Unchanged
3	Change in motor unit and type II fiber atrophy	40	Worse
4	Myopathic changes with whorled and ring fibers	45	Worse
5	Chronic change in motor unit and some mitochondrial alterations	60	Unchanged
6	Severe type II fiber atrophy	35	Severe type II fiber atrophy and severe myopathic change
7	Chronic neurogenic signs	80	Desmin accumulation
8	Normal	60	Normal
9	Slight variation in fiber size and mitochondrial signs	105	Unchanged
10	Neurogenic signs	35	Unchanged
11	Slight neurogenic signs	15	Worse

subclinical diabetes, and previous chemotherapeutic treatments (Table 1).

In five biopsies out of eleven performed after HLP, the histological picture was changed: in three patients neurogenic damage was revealed by an evident type-grouping and in two patients, both treated with melphalan (nos. 4 and 6), myophatic damage occurred. However, no evident clinical signs were present. No change occurred in the patient with normal pre-HLP biopsy.

A significant reduction ($P < 0.0001$) in the diameter of type I and type II fibers was present in nine cases, with no difference between patients treated with doxorubicin or melphalan. In Table 3 the direction and magnitude of the variation in fibers size are listed; in particular, there was a huge reduction in type II fiber diameter observed in patient no. 7. In this patient the muscle biopsy before HLP showed a slight change in fiber size with some target fibers and evident type-grouping with ATPase stain. These findings were compatible with chronic neurogenic alterations, which can probably be ascribed to subclinical diabetes. Muscle biopsy performed 80 days after HLP showed a considerable accumulation of desmin inside the muscular fibers. Desmin accumulation, while positive for α-actinin and titin (Fig. 1), was negative for vimentin. Spectrin showed integrity of muscle membrane. A fiber count revealed a highly significant reduction in the diameter of type II fibers ($P < 0.0001$).

Discussion

The use of heat as a treatment for cancer began in 1866 when Bush reported a curious case of sarcoma of the face that disappeared after fever caused by erysipelas [3]. Unfortunately, the therapeutic efficacy of hyperthermia is somehow limited by the thermoresistance of some tumor cells.

Several in vitro and in vivo studies have demonstrated that the contribution of hyperthermia and chemotherapy can promote a synergistic effect [9, 10]. Searching for factors to condition the efficacy of HLP, it has also been shown that the best results can be achieved by a perfusion temperature of 41.5 °C or higher, and standard or even higher drug concentration [5]. However, when these conditions are reached, some adverse effects may appear whose causes remain to be clarified.

Neurotoxicity is one of the most troublesome side effects and has been reported to occur in from between 1 and 48% of the patients undergoing HLP [25]. Neurotoxicity may be the result of several effects, such as temperature, the drug used, the compartmental syndrome [22] or, most often, the pressure-induced

Table 3 Type I and type II fiber diameters (expressed in μm ± SD) of muscle fibers before and after limb surgery

Patient	Type I-pre	Type II-pre	Type I-post	Type II-post	Drug
2	72.8 ± 19.9	66.15 ± 10	59.84 ± 11.84**	42.4 ± 13.38**	Melphalan
3	64.56 ± 11.1	49.85 ± 10.95	56.23 ± 12.57**	48.39 ± 11.22	
4	66.77 ± 12.51	70.14 ± 12.92	57.2 ± 14.08**	56.23 ± 14.26**	
6	65.18 ± 11.88	47.76 ± 18.76	64.95 ± 11.6	45.06 ± 14.56	
10	60.67 ± 13	30.57 ± 14.57	63.8 ± 13.4	40.64 ± 12.59**	
11	74.2 ± 24.25	64.18 ± 21.65	72.9 ± 19.36	80.65 ± 26.41*	
1	56.64 ± 13.9	39.8 ± 15.67	59.26 ± 13.18	39.12 ± 15.08	Doxorubicin
5	69.01 ± 18.85	47.38 ± 11.05	69.68 ± 12.97	55.49 ± 14.1	
7	52.5 ± 11.2	58.96 ± 16.3	45.7 ± 14.7*	17.52 ± 11.18**	
8	59.24 ± 10	66.92 ± 12.57	57.28 ± 10.46	57.34 ± 8.79**	
9	76.8 ± 15.67	48.8 ± 12.65	66.5 ± 16.8**	47 ± 15.6	

* $P < 0.001$; ** $P < 0.0001$

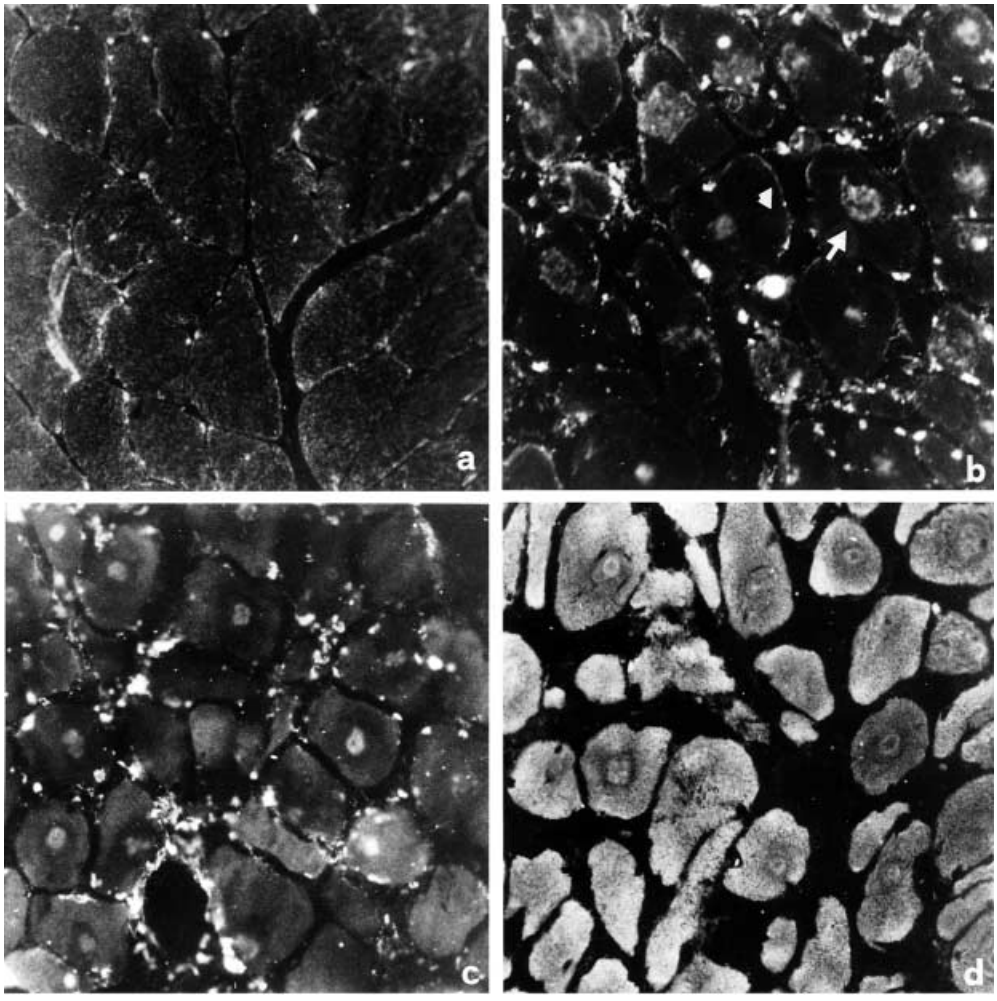

Fig. 1 **a** Immunohistochemistry with anti-desmin antibodies in the biopsy of patient no. 7 before HLP does not show any accumulation of desmin. **b** After HLP marked desmin-positive accumulations are visible in almost all fibers (*arrow*). **c** The same accumulations are positive with anti-α-actinin antibody. **d** Immunohistochemistry with anti-titin antibody shows a halo of disorganization of the intermyofibrillar network surrounding a titin-positive central core

damage ensuing from a too tightly applied isolating bandage, especially at the auxiliary level [25]. Temperature is crucial in determining tissue damage: for example, it is well known that synergism between melphalan and hyperthermia occurs at 42 °C, albeit this temperature is too harmful for limb tissues. A temperature of 41.5 °C is considered a good compromise between toxicity and effectiveness since no difference in systemic or local toxicity was observed when higher temperatures and drug concentrations were employed, provided that the perfusion temperature did not exceed 42 °C [4, 5].

Different degrees of limb reaction may also result from deterioration of the blood gas value of the perfusate, low flow rate, high total melphalan dosage, high melphalan peak concentration, and a large area under the melphalan concentration curve [26]. On the other hand, no report has definitely proved the direct neurotoxic effect of melphalan. When TNFα is added to melphalan a greater rate of remission is reached without an increase in the overall and neuromuscular toxicity [6, 23].

Contrary to the findings of an unacceptable regional toxicity after doxorubicin-HLP, not linked to drug concentration [12], a more recent study demonstrates that doses of doxorubicin between 0.7 and 1.4 mg/kg, as we used in our patients, can be safely administered at a mean temperature of 41.5 °C [21].

As shown in Table 2, three of our patients showed a worsening in neurogenic damage, albeit a close monitoring of the condition involved in neurotoxicity, i.e. drug dosage and perfusion temperature, and the fact that all the procedures were performed in the lower limbs. All three of these patients had subclinical signs of neuropathy before the HLP; type II atrophy, observed in several muscular specimens, could be due either to previous steroid therapy or immobilization, while minor mitochondrial abnormalities could be related to the age of the patients. A myopathic pattern was present in two patients, but one of them was already showing muscular damage in the biopsy before treatment.

Previous studies [13, 22, 25, 26] pointed out that toxic effects observed in neuromuscular systems could be related to the HLP conditions, in particular perfusion temperature or the type of drug. Besides the technical parameters of HLP, an impaired neuromuscular system before surgery could play a role in the incidence of neurotoxic effects.

The large range of time between the primary and second intervention could be a disadvantage in the comparison of the histopathological effects particularly on regeneration phenomena, less on the type and entity of the muscle damage. In fact, there is no correlation between the lapse of time and the histopathological alterations.

Our study shows that the condition of the neuromuscular system before the procedure is critical in determining HLP toxicity. It is well known that young patients with good limb musculature better tolerate higher doses than those with flabby muscles and increased fatty tissue [13]. This is consistent with the observation of normal histological pictures before and after HLP in our 20-year-old patient (no. 8).

Besides the evident reduction in type II fiber diameter, patient no. 7 exhibited an evident accumulation of desmin within the muscle fibers in the biopsy performed after HLP with doxorubicin. The relationship between desmin accumulation and doxorubicin cardiomyotoxicity has to be clarified because no other doxorubicin-treated patient showed a similar finding.

A previous report suggested a large interindividual variation in doxorubicin metabolism, possibly due to a genetic polymorphism [1]. Doxorubicin myotoxicity is well known and it has been taken advantage of it in the treatment of blepharospasm, hemifacial spasm [28] and, more recently, cervical dystonia [19].

In many in vivo and in vitro studies, doxorubicin (dox) causes myofibrillar loss in a dose-dependent manner [18, 20] with disorganization of Z-band structure and thin filament disarray [17]. Many mechanisms of action have been implicated in dox-induced cardiomyotoxicity, such as free-radical stress, increase in contractile force or suppression of transcripts encoding myofibrillar proteins and energy production proteins with ATP depletion and myofibrillar degeneration in cardiomyocites [11], but the exact mechanism of action is still unknown.

Three hypotheses have been suggested to explain its mechanism of action. The first suggests an inhibition of myoblast fusion and accumulation of muscle-specific transcripts by inhibition of MyoD activity through, at least in part, induction of Id gene expression [14], probably as a transcriptional induction of a response to DNA damage. In cardiomyocytes, it has been demonstrated that doxorubicin suppresses transcripts that encode for myofibrillar proteins and energy production proteins with ATP depletion and myofibrillar degeneration [17]. The second hypothesis supports an oxidative stress on cardiac myocytes by reactive oxygen species generated by the stable complex of anthracyclines with iron. It has been demonstrated that free-radical scavengers and metal-chelating agents have the ability to reduce the toxicity of cardiomyocytes. The third proposes an increase in the maximal tension in cardiac muscle fibers by direct interaction with the actin-myosin cross-bridges [2].

Desmin is an insoluble cytoplasmic filament that belongs to class III or the vimentin-like intermediate filaments and is located in the Z-band region. An insoluble skeleton composed of intermediate filament remains after extraction with non-ionic detergents and a high concentration of KI that solubilizes other contractile elements [16, 24].

Desmin myopathies are a large and heterogeneous group of diseases, sometimes on an hereditary basis, in which desmin accumulation is the main pathological feature. Often desmin accumulation stains positively with anti-ubiquitin antibodies. Interestingly, many of the patients with desminopathy presented a hypertrophic cardiomyopathy, similar to that induced by doxorubicin [8]. We speculate that collapse of the desmin skeleton could be the final common pathway in the dox-induced cardiomyopathy after destruction of myofibrillar filaments. In fact the term myofibrillar myopathy has also been applied to desminopathies. The failure to metabolize desmin through the ubiquitin system could activate free radicals yielding secondary damage. In our patient the lisosomal elimination of muscle proteins, usually efficacious, was not effective for an unknown reason, inducing a secondary desminopathy.

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