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● *Original Contribution*

THE EFFECTS OF SUCCESSIVE HIGH-ENERGY SHOCK-WAVE TUMOR ADMINISTRATION ON TUMOR BLOOD FLOW

E. B. CORNEL,[†] G. A. H. J. SMITS,[†] F. M. J. DEBRUYNE,[†] A. HEERSCHAP,[‡]
 J. A. SCHALKEN[†] and G. O. N. OOSTERHOF[†]

Department of [†]Urology and [‡]Radiology, University Hospital, Nijmegen, The Netherlands

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Abstract—The effects of repeated high-energy shock wave (HESW) tumor administration on tumor blood flow (TBF) were studied in NU-1 human kidney cancer xenografts. Deuteriated water was used as a magnetic resonance spectroscopic detectable tracer for measuring tumor blood flow. Tumors were exposed twice to 800 electromagnetically generated HESW, with a 24-h interval or sham exposed. No changes in TBF occurred after sham exposure to HESW. TBF levels 2 h after the first and second HESW application were, respectively, 46% and 37% lower than the mean preexposure TBF value and returned to normal levels within 16 h. There was statistically no difference found between the effects on tumor blood flow after the first and second HESW exposure. These observations are in agreement with earlier studies and provide a rationale to shorten the time interval between HESW monotreatments to 2 to 3 h.

Key Words: High-energy shock waves, Nuclear magnetic resonance spectroscopy, Cancer xenografts, Tumor blood flow.

INTRODUCTION

Experimental animal studies, using different tumor model systems, have shown that temporal growth suppressive effects can be achieved by treating tumors with high-energy shock waves (HESW) (Debus et al. 1989, 1991; Hoshi et al. 1991; Oosterhof et al. 1990b; Russo et al. 1985; Weiss et al. 1990). Moreover, combination of HESW with chemotherapy or cytokines resulted in additive or synergistic antitumor effects (Lee et al. 1990; Oosterhof et al. 1990a, 1991). A number of variables that influence these antitumor effects have been identified, including tumor vascularization, time interval between successive treatments, number of HESW, number of HESW foci, tumor size and tumor model system (Hoshi et al. 1991; Oosterhof et al. 1990b; Weiss et al. 1990).

Several studies have been performed to clarify the mode of action of HESW. Gamarra et al. (1993) demonstrated, with the use of autoradiography of iodo-¹⁴C antipyrine, a temporary reduction of the perfusion of A-mel-3 tumors after treatment with HESW. Using

magnetic resonance spectroscopy (MRS) techniques we found a temporary impairment of tumor blood flow (TBF) in the NU-1 renal cancer xenograft after HESW application, resulting in a metabolic inactivation and acidification of the tumor (Smits et al. 1991, in press). Histological studies of this NU-1 tumor show changes in tumor vasculature due to HESW exposure, *i.e.*, disrupted capillaries, extravasation of erythrocytes, distended microvessels with tightly packed erythrocytes and thrombi formation (Oosterhof et al. 1990a). Apparently, one of the main targets of HESW is the vascular system (Debus et al. 1991; Gamarra et al. 1993; Hoshi et al. 1991; Smits et al. 1991; Smits et al. in press; Oosterhof et al. 1990b; Russo et al. 1987).

As mentioned above, earlier studies using different tumor models revealed that the antitumor effects of HESW could be enhanced by repeated HESW tumor treatments and that shortening of the interval between subsequent HESW treatments from 24 h to 5 h leads to more effective tumor growth inhibition (Oosterhof et al. 1990b; Weiss et al. 1990). It is tempting to speculate that increased efficacy is associated with the extent and duration of the decrease of TBF after successive HESW exposures.

We therefore evaluated the effects of repeated HESW application on TBF. As in our previous MRS

Address correspondence to: Dr. G. O. N. Oosterhof, M.D., Ph.D., Department of Urology, University Hospital, Geert Grooteplein Zuid 16, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

study, intratumoral injected deuteriated water (D_2O) was used as a MRS detectable tracer for measurement of TBF (Smits et al. in press). This technique has proved to be a sensitive and reproducible method in the determination of TBF and perfusion (Evelhoch 1992; Kim and Ackerman 1990; Larcombe-McDouall and Evelhoch 1990; Neil 1991). Moreover, since the MRS TBF measurement method is nondestructive and relatively noninvasive, it is possible to measure TBF in the same subject repeatedly. It is therefore an ideal technique to monitor changes in tumor blood flow after repeated HESW tumor administrations.

MATERIALS AND METHODS

Animals

Xenografts were transplanted in 6- to 8-week-old male BALB/c athymic mice (Bornholtgård, Ry, Denmark). The mice were kept in groups of 5 in PAG type 2 cages covered with an iso cap (Iffa Credo, France) for sterile conditions. The mice were fed *ad libitum* with irradiated SRM-A MM food (Hope Farms, Woerden, The Netherlands) and drinking water was acidified (pH 3).

Tumors

The human renal cell carcinoma NU-1 xenograft was established in our laboratory by subcutaneous implantation of small tumor pieces derived from a tumor nephrectomy specimen (Oosterhof et al. 1990a). For the HESW studies, tumors were implanted subcutaneously as trocar pieces at the upper part of the hind limb. Passages 9 to 14 *in vivo* were used. The three dimensions of the tumor were determined with a precision sliding caliper by measuring the maximum diameter and the diameters perpendicular to it. The tumor volume was calculated by the equation $(l \times w \times h) \times (\pi/6)$. Tumors were allowed to grow to a volume of 300 to 350 mm³ (mean 325 mm³).

HESW

HESW were generated by an experimental set up of the commercially available electromagnetic shock-wave source Lithostar Plus (Siemens AG, Erlangen, Germany). The physical and technical characteristics of this experimental shock-wave source have been described recently (Steinbach et al. 1992). In short, the positive pressures range from 24 to 63 MPa, the corresponding energy densities, defined as the time integral over the duration of a pulse, range from 0.08 to 0.6 mJ/mm². The main frequency of the pulse was 200 kHz (resonance frequency of the system), but frequencies up to 10 MHz are included. Negative pressures range from 5 to 10 MPa. The pressure rise time, defined

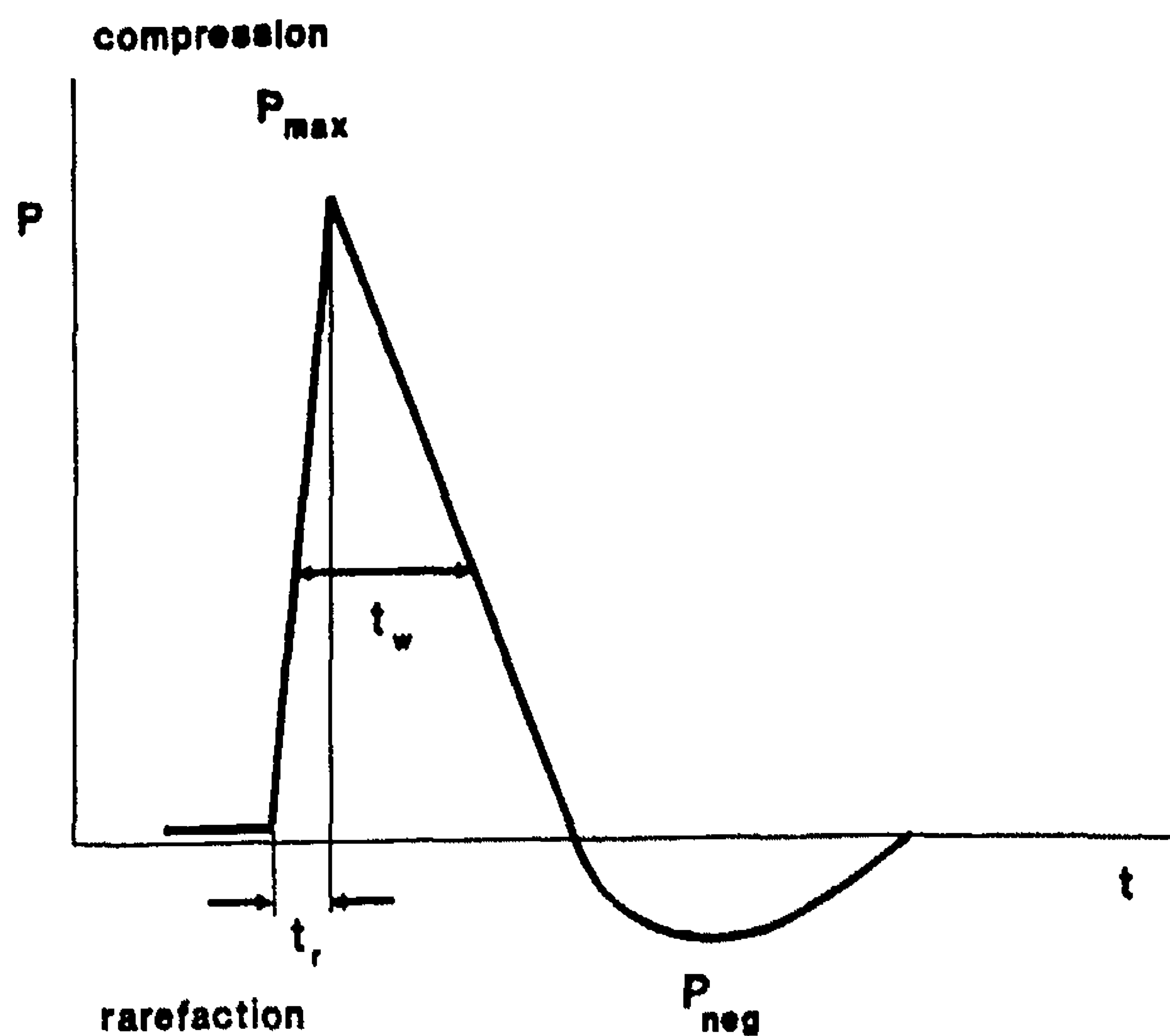


Fig. 1. Parameters of a focused shock wave. Pressure maximum (P_{max}), pressure rise time (t_r), half-width time (t_w) and negative peak pressure (P_{neg}).

as the time for the pressure to rise from 10% to 90% of the value of P_{max} , ranges from 30 to 120 ns. The half-width time, defined as the half-amplitude width of the initial positive pressure half cycle, is about 5 μ s. A graph of the pulse shape is shown in Fig. 1. The pulse-to-pulse variation was about 2% to 3%. The HESW were focused centrally on the tumors. In this way we ensured that, according to pressure measurements, all parts of the tumors which had diameters of less than 8.5 mm experienced more than one third of the maximal pressure applied centrally. The shock waves were applied with a frequency of 2 Hz.

Just before HESW (sham) exposure, mice were anaesthetized with ketamine hydrochloride (Ketalar, Parke-Davis), 100 mg/kg, for a 15-min period. Mice were kept in fixed position in a plastic tube which was placed in the water bath. The tumor bearing leg was projected through a hole in the base of the plastic tube and the center of the tumor was positioned in the focal area through a three-dimensional positioning system. Sham exposure was achieved by placing the tumor in such a way that the focus (as determined by pressure measurements) did not encounter the tumor. Each HESW administration consisted of, in total, 800 pulses with an energy density of 0.47 mJ/mm² (pulse repetition frequency of 2 Hz). All experiments were carried out with degassed water at 37°C. The NU-1 tumors were exposed twice to HESW (at $T = 0$ and $T = 24$ h).

NMR spectroscopy

In vivo NMR spectroscopy was performed on a 4.7-T Bruker WM-200 spectrometer as described pre-

viously (Smits *et al.* 1991). The mice were anaesthetized by means of a gas flow of 1.5% enflurane in a O₂/N₂O mixture applied through a nose cone. Body temperature was monitored using a nonmagnetic temperature probe and maintained at 37°C by a flow of humidified warm air. The tumors were exposed through a matched hole in a Faraday shield and positioned partly inside the radio-frequency (RF) coil to prevent any contribution of signals from surrounding tissue. The magnetic field homogeneity was optimized by using the ¹H NMR signal from tumor H₂O. Typical linewidths of this signal were 0.1 to 0.2 ppm. The animals were spectroscopically examined 2 h before the first administration of HESW ($T = -2$) and at $T = 2$, $T = 8$, $T = 16$, $T = 26$, $T = 32$ and $T = 40$ h.

The deuterium measurements were performed at a resonance frequency of 31 MHz, employing a home-built probe with a 1H/2H/31P triple tunable three-turn solenoid coil with an inner diameter of 14 mm. After optimizing the field homogeneity the RF probe was removed from the magnet. According to the results of Larcombe-McDouall and Evelhoch (1990), 60 to 70 μ l PBS-D₂O was injected (concentric) intratumorally at three different sites, using a 30-gauge microsyringe. During this procedure, the tumors remained in their original position in the RF coil. The probe was repositioned in the magnet and serial ²H NMR measurements were started within 70 s after injection.

Sixty-four deuterium spectra were collected in 49-s time blocks with 64 scans/block using a 10- μ s RF pulse, 2 k data points and a spectral width of 5000 Hz.

Data analysis

For each timepoint, every first and following fifth deuterium spectrum recorded was evaluated employing the NMR1 package (New Methods Research, Inc., Syracuse, NY) on a Sun Sparc 330 station (Sun Microsystems, Inc., Mountain View, CA). Free induction decays (FIDs) were Fourier transformed and the phase was manually corrected. Deuterium resonances were semi-automatically fitted to Lorentzian lineshape model functions. Absolute integrals of the deuterium resonance were calculated and plotted as a function of time. The D₂O clearance curves thus obtained are presumed to represent blood flow within the whole tumor (Larcombe-McDouall and Evelhoch 1990).

Clearance curves of D₂O were best fitted using a single compartment model. Tumor blood flow was thus determined using a single compartment model as described by Kim and Ackerman (1990). In all cases, the deuterium residue washout could accurately be described by:

$$Q(t) = Q_{bg} + [Q(0) - Q_{bg}] \times \exp(-kt).$$

This equation describes the simplest first order kinetic "single compartment model," where $Q(t)$ is the tracer quantity at time t , Q_{bg} is the nonzero steady-state tracer background, $Q(0)$ is that at $t = 0$ and k is the first order rate constant governing tracer washout (Kim and Ackerman 1990).

Since the steady-state tracer background (residual from previous experiment) has already been subtracted from the original decay curve the deuterium washout decay can be formulated as:

$$Q(t) = Q(0) \times \exp(-kt).$$

The formula $TBF = 100 \times \lambda \times k$ is then an estimate of the initial tumor blood flow (in units of milliliters per 100g \times min). Here, λ is the tumor-to-blood partition coefficient (ratio of the water weight of a unit mass of tumor to the water weight of a volume of blood) which was determined by measuring wet and dry weights of tumor and blood. Dry weights were achieved by drying in an oven at a temperature of 90°C until no changes in weight were measured. λ was calculated to be 0.906 ± 0.019 (in units of milliliters per gram per hour) ($n = 5$).

Statistics

Because of the normal distribution pattern of the calculated TBF, the two-sided t test was used to compare the TBF between the control and HESW applied group and to evaluate the effect of the second HESW administration. Statistical significance was set at $p < 0.05$.

RESULTS

Although it is difficult to define a specific biological active focus, and thus to define an effective treatment volume, all tumor tissue was exposed to at least one third of the maximum positive pressure applied. Earlier studies have shown that this way of HESW tumor exposure leads to significant antitumor effects evaluated for tumors as a whole (Debus *et al.* 1989, 1991; Hoshi *et al.* 1991; Lee *et al.* 1990; Oosterhof *et al.* 1990a, 1990b, 1991; Russo *et al.* 1985; Weiss *et al.* 1990).

According to the results of Larcombe-McDouall and Evelhoch (1989), 60 to 70 μ L D₂O was injected intratumorally at three different sites, presuming that the D₂O clearance curves thus obtained represent blood flow within the whole tumor. The D₂O clearance curves were best fitted using a one-compartment model.

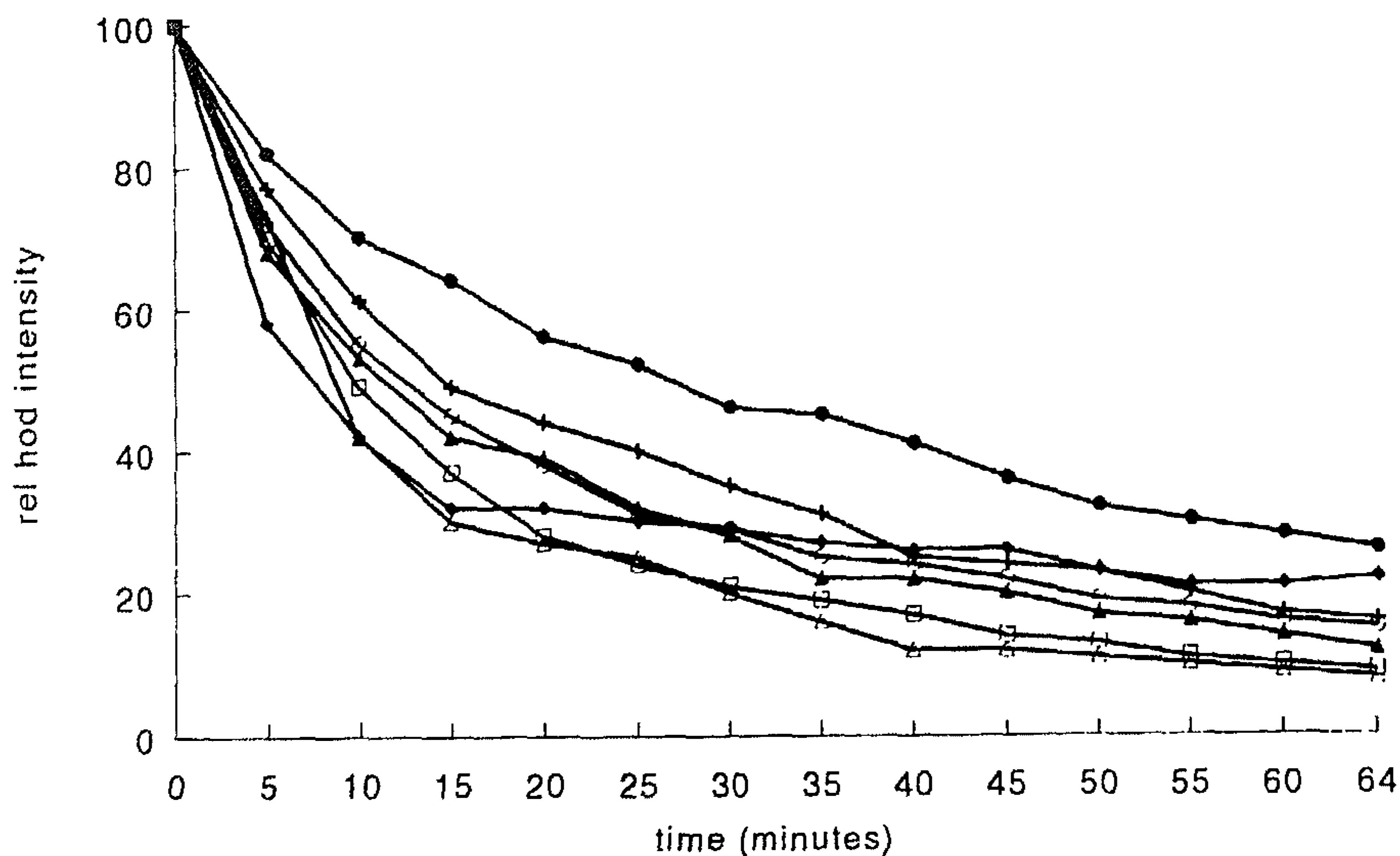


Fig. 2. D_2O outflow curves, with data points each five measurements, at seven different time points of a sham-treated control tumor obtained by 2H MRS after intratumoral injection of an isotonic D_2O solution. Closed circle: pre-exposure; open triangle: $T = 2$ h; open circle: $T = 8$ h; cross: $T = 16$ h; closed triangle: $T = 26$ h; open square: $T = 32$ h; closed square: $T = 40$ h.

Tumor blood flow in control tumors

In Fig. 2 seven D_2O outflow curves obtained at $T = -2$, $T = 2$, $T = 8$, $T = 16$, $T = 26$, $T = 32$ and $T = 40$ h from one representative control NU-1 tumor are shown. Calculated mean TBF of all HESW nonexposed control tumors ($n = 5$) for the subsequent time points revealed no significant changes in TBF during this time period ($p = 0.17$, Fig. 3).

Tumor blood flow in HESW-exposed tumors

The mean pre-HESW (sham) exposure TBF values in the HESW and control groups were not statistically

different (Fig. 3). Administration of 800 HESW on the center of the tumor resulted in a decrease of the mean TBF of $46 \pm 19\%$ after 2 h and $16 \pm 30\%$ after 16 h. A second HESW exposure, 24 h after the first, resulted in a decrease of the mean TBF with $37 \pm 23\%$ after 2 h and $16 \pm 34\%$ after 16 h when compared to preexposure values. As a result, TBF levels 2 h after the first and second HESW administration were significantly lower than the preexposure TBF ($p = 0.02$ and $p = 0.04$, respectively). Moreover, comparing the effect on TBF of the first and second HESW administration revealed no significant difference ($p = 0.45$).

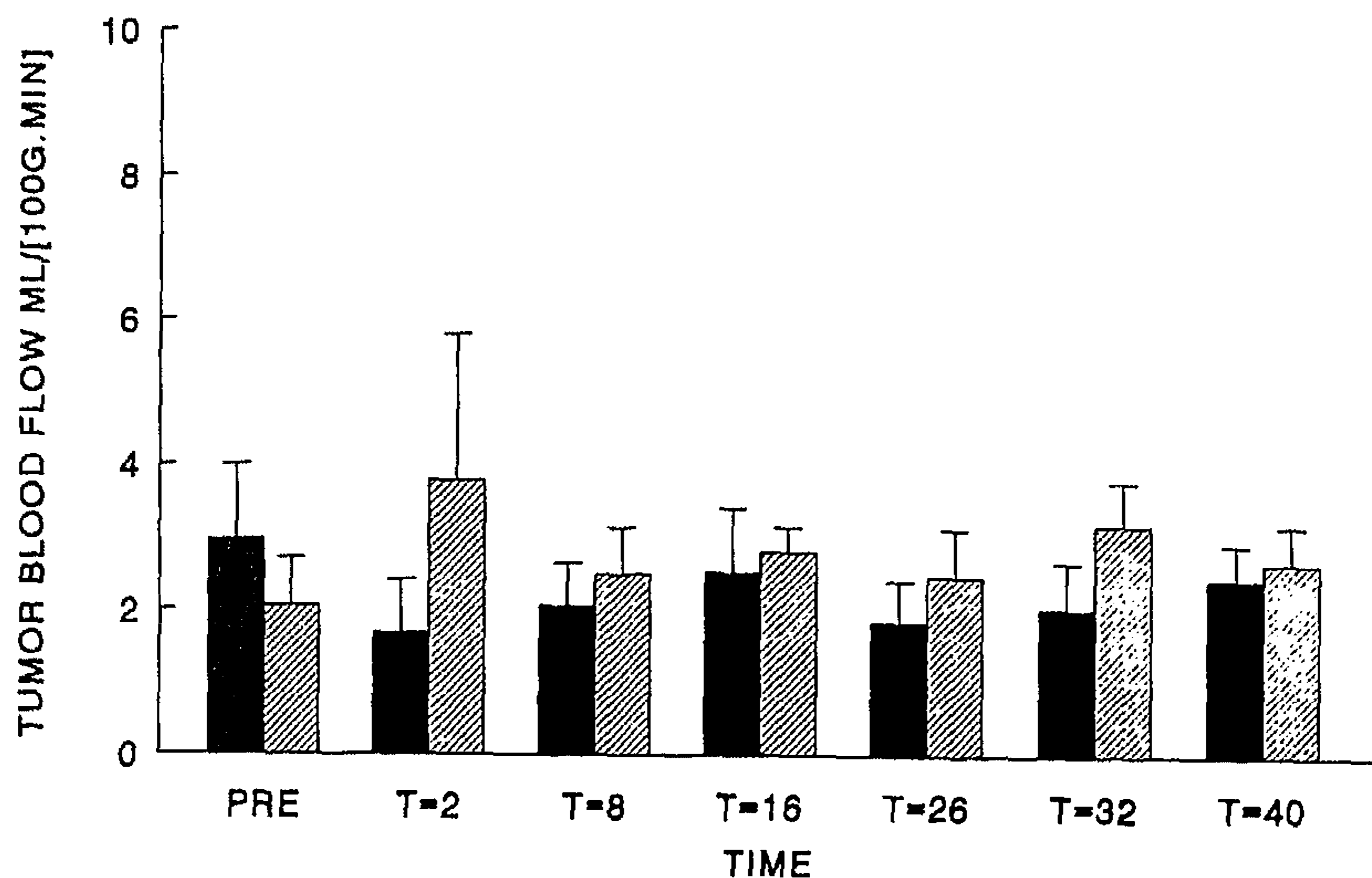


Fig. 3. Calculated mean tumor blood flow (in units of $ml/100\text{ g} \times \text{min}$) with SD of five sham-treated control tumors (gray bars) and of five HESW-treated tumors (black bars). Tumors were HESW- or sham-treated at $T = 0$ and at $T = 24$ h.

DISCUSSION

Recently, it has been demonstrated that the vascular functionality of tumors is the primary target of HESW tumor therapy (Gamarra *et al.* 1993; Smits *et al.* in press). Exposure of tumors to HESW causes a temporary decrease in TBF, resulting in a metabolic inactivation and acidification (Smits *et al.* 1991, in press). The modulatory effects of HESW on TBF may be used to overcome the limited efficacy of other treatment modalities. For instance, a threefold increase of the intratumor concentration of systemically given tumor necrosis factor alpha (TNF- α) was achieved after HESW tumor treatment (Cornel *et al.* in press). Moreover, the HESW-induced decrease of TBF may probably improve the efficacy of thermotherapy. Monitoring the effects on TBF of (repeated) HESW exposure(s) is therefore of importance to improve the efficacy of HESW treatments, not only as monotherapy but also in combination with other therapies. A 24-h time interval was chosen because we wanted to investigate the effects of a second HESW treatment after functional recovery of the TBF.

Several methods can be used to measure blood flow and tissue perfusion, either directly or indirectly (Appelgren 1979). Among the methods developed to measure TBF indirectly, radiolabeled and MRS detectable tracers are most commonly used. There are several advantages of MRS-based methods above the use of radiolabeled tracers (Evelhoch 1992). First, MRS is nondestructive and relatively noninvasive. Second, stable isotopes (*e.g.*, deuterium or fluorine) are used, eliminating problems of radiation exposure and special tracer handling. Finally, concurrent ^1H and ^{31}P MRS measurements can be performed, evaluating tissue metabolism. On the other hand, the main disadvantage of this technique is that it requires specialized and expensive equipment. In the present study we used the deuterium clearance method to calculate TBF at various timepoints after successive HESW exposures. In an earlier study we demonstrated that in the NU-1 tumor this method gives reproducible deuterium outflow curves after repeated intratumoral D_2O injections, independent of the number of injection sites (Smits *et al.* in press). The maximal mean absolute difference found between washout curves, obtained at time intervals between 3 and 6 h for an individual tumor, was 7% of the relative HOD intensity at $t = 0$ (Smits *et al.* in press). Here we demonstrate that a second HESW application of this NU-1 tumor results in a decrease in tumor blood flow which is statistically not different from the effect on TBF seen after the first HESW exposure. Both HESW exposures resulted in a significant decrease of TBF 2 h after therapy, followed by

normalization to preexposure values within 16 h. The extent and duration of the decrease in TBF after the first HESW administration were comparable with those described earlier (Smits *et al.* in press). However, the absolute TBF flow values of the NU-1 tumors used in this study were much lower than found earlier (Smits *et al.* in press). Several reasons can be taken into consideration for this major discrepancy. First, there are major differences in tumor doubling time between the early passages used now and the late passages used earlier (8 and 3.5 days, respectively). The lower tumor doubling time of these early passages of the NU-1 tumor might well be representative of a less well-developed vascular system. Second, since the MRS surface coil used in the present study was bigger than used in our earlier work, the tumors needed to grow to significantly higher volumes compared to the aforementioned study. For other xenografts it has been demonstrated that TBF values correlate with tumor volume, the bigger the tumor the lower the TBF (Lyng *et al.* 1992). Finally, in contrast to our earlier study, D_2O clearance curves were best fitted using a one-compartment model. For the RIF tumor it has been demonstrated that TBF values, calculated using a monocompartment model, were significantly lower compared to the TBF values calculated from the same clearance curves, using a two-compartment model (Kim and Ackerman 1988).

Earlier studies, using different tumor models, revealed that the antitumor effects of HESW could be enhanced by repeated HESW tumor treatments and that shortening of the interval between subsequent HESW treatments leads to more effective tumor growth inhibition (Oosterhof *et al.* 1990b; Weiss *et al.* 1990). Although the exact length of the time interval remains debatable, this study further rationalizes the shortening of the time interval between HESW exposure. Reperfusion of the HESW-exposed tumor cannot occur and the prolonged ischemia will induce more cell death. Denekamp *et al.* (1983) reported for the SA F mouse tumor that 15 h of ischemia was required to obtain local cure of the tumor. Assuming that such a long time period will also be necessary for other tumors, this would imply that five or six successive HESW treatments should be given to achieve sufficient ischemia time to provoke complete local tumor regression.

Optimization of time schedules for HESW treatment in combination with other therapies, *e.g.*, systemically given drugs (chemotherapy or BRMs), based on this study, will be more difficult. Recently we demonstrated that a threefold increase in the intratumor concentration of systemically given TNF- α can be achieved after one HESW tumor treatment (Cornel *et al.* in press). Yet it is not known to what extent TBF

must be recovered before a second treatment of HESW in combination with systemically given drugs would be effective. It is obvious that drugs need to have access to the tumor before the selective shutdown of the tumor vasculature will result in increased intratumoral drug levels.

In conclusion, it becomes clear that HESW are capable of damaging the tumor vasculature selectively. The reason for the discrepancy between the histologically proven vascular damage and the relatively short functional effects on tumor blood flow is not yet known. It might be possible that after HESW exposure a temporary (cavitation induced) increased vascular permeability occurs, resulting in the extravasation of the erythrocytes.

It is tempting to speculate that this HESW-induced decrease of tumor blood flow will be able to rule out problems of inadequate drug uptake and non-optimal distribution in the tumor. The potential of HESW to increase the efficacy of systemic therapies by selective temporary changes in TBF will possibly determine its clinical application.

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