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Tumour pO_2 can be increased markedly by mild hyperthermia

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Summary We have studied the feasibility of improving tumour oxygenation with hyperthermia at modest temperatures which are achievable with the use of presently available clinical hyperthermia machines. FSaII tumours grown s.c. in the leg of C3H mice and R3230 AC tumours grown s.c. in the leg of Fischer rats were heated with a water bath and the tumour pO_2 was determined with an Eppendorf pO_2 histograph. The median pO_2 in 7-8 mm diameter control FSaII tumours was 6.5 ± 0.5 mmHg and it increased to 16.6 ± 1.1 mmHg when the tumours were heated at 41.5°C for 1 h. The median pO_2 in 10 mm diameter control R3230 AC tumours was 3.7 ± 0.3 mmHg. Heating at 42.5°C for 30 min increased the median pO_2 in the R3230 AC tumours to 12.2 ± 1.8 mmHg. The pO_2 in FSaII tumours measured 24 h after heating at 41.5°C for 1 h was still higher than the pO_2 before heating. The % frequency of pO_2 values lower than 5 mmHg decreased markedly when the tumours were heated at the modest temperatures mentioned above. Modest temperature hyperthermia (MTH) may be an efficient and useful means to improve the oxygenation of human tumours.

Keywords: tumour oxygenation; hypoxia; oxygen electrode

Increasing evidence suggests that varying fractions of viable cells in human tumours are hypoxic and are thus resistant to radiotherapy (Dische, 1991). Therefore, numerous means have been proposed and tested to increase oxygenation in human tumours. Among the many approaches, breathing hyperbaric oxygen (Dische, 1991) or carbogen (Siemann *et al.*, 1977; Song *et al.*, 1987), substituting blood with Fluosol DA (Song *et al.*, 1987; Teicher and Rose, 1984) and systemic administration of nicotinamide (Horsman *et al.*, 1987; Siemann *et al.*, 1994) or pentoxifylline (Honess *et al.*, 1995; Song *et al.*, 1992) have been demonstrated to improve oxygenation in rodent tumours as well as in some human tumours. However, the clinical results of using these approaches have been far from satisfactory. The present study was undertaken to investigate whether tumour oxygenation can be increased by increasing tumour blood flow by modest thermal doses.

It is well-known that the newly formed blood vessels in tumours are structurally immature and are thus more vulnerable to heat compared with the blood vessels in normal tissues (Dewhirst *et al.*, 1984; Jain and Ward-Hartley, 1984; Reinhold and Endrich, 1986; Song, 1984; Vaupel, 1990). Therefore, most of the previous clinical hyperthermia protocols were designed to exploit the preferential vascular damage in tumours in addition to causing direct killing of tumour cells and also directly radiosensitising tumour cells. Unfortunately, currently available hyperthermia devices have been ineffective in raising the temperature of human tumours high enough to cause the aforementioned effects. Oleson (1995), therefore, hypothesised that in previous clinical studies in which hyperthermia was shown to improve the effect of radiotherapy, hyperthermia might have improved tumour oxygenation, and thus indirectly radiosensitised tumours through an increase in tumour blood flow. Although we and other investigators have indeed observed that heating tumours to modest temperatures causes increases in tumour blood flow, the possible use of hyperthermia to increase tumour pO_2 has not yet been investigated systematically. In the present studies, we determined the effects of hyperthermia at modest temperatures on the pO_2 in FSaII tumours in C3H mice and in R3230 AC tumours in Fischer rats.

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Materials and methods

Tumours

FSaII tumours Early generations of this fibrosarcoma of C3Hf/Sed mice were obtained from the laboratory of Dr H Suit, Massachusetts General Hospital, Boston, MA, and are stored in liquid nitrogen in our laboratory (Song *et al.*, 1992). Every 3-4 months, the stock cells are thawed and cultured in RPMI-1640 medium supplemented with 10% calf serum. For the present studies, the cells in exponential growth phase in culture were trypsinised, washed and about 2×10^5 cells in 0.05 ml medium without serum were injected subcutaneously (s.c.) into the right hind limbs of male C3H mice. The tumours grew to 7-8 mm in diameter in 8-10 days when they were used to study the effect of hyperthermia on tumour oxygenation.

R3230 AC tumours The stock cells of this mammary adenocarocinoma of Fischer rats were obtained from Dr M Dewhirst, Duke University, Durham, NC, and are stored in liquid nitrogen in our laboratory. Every 6 months, the frozen tumour cells are thawed and maintained *in vivo* by serial transplantation (Hasegawa and Song, 1991). For experiments, the tumours grown in the flank of rats were dissected, and about 2 mm^3 tumour piece was s.c. injected into each right leg of 300-325 g male Fischer rats with the use of trocar needles. This tumour grows slowly and experiments were performed 20-30 days after tumour inoculation when the tumours had grown to about 1 cm in diameter.

Heating of tumours

FSaII tumours The tumour-bearing mice were anaesthetised by an intraperitoneal (i.p.) injection of a mixture of ketamine and xylazine at 10 mg kg^{-1} and 1 mg kg^{-1} respectively. The hairs on the tumour-bearing legs were clipped with an Electrical clipper, the mice were then placed on specially designed Plexiglas jigs and the tumour-bearing legs were extended and anchored vertically to a support on the jig using surgical silk. The anchored legs were immersed vertically into a preheated water bath, as we described previously (Song *et al.*, 1994). The temperature in the tumours was measured using a 29-gauge, needle-type thermocouple. The tumour temperature was 0.1-0.3°C lower than the temperature of the heating water.

R3230 AC tumours The tumour-bearing rats were anaesthetised by an i.p. injection of a mixture of ketamine and

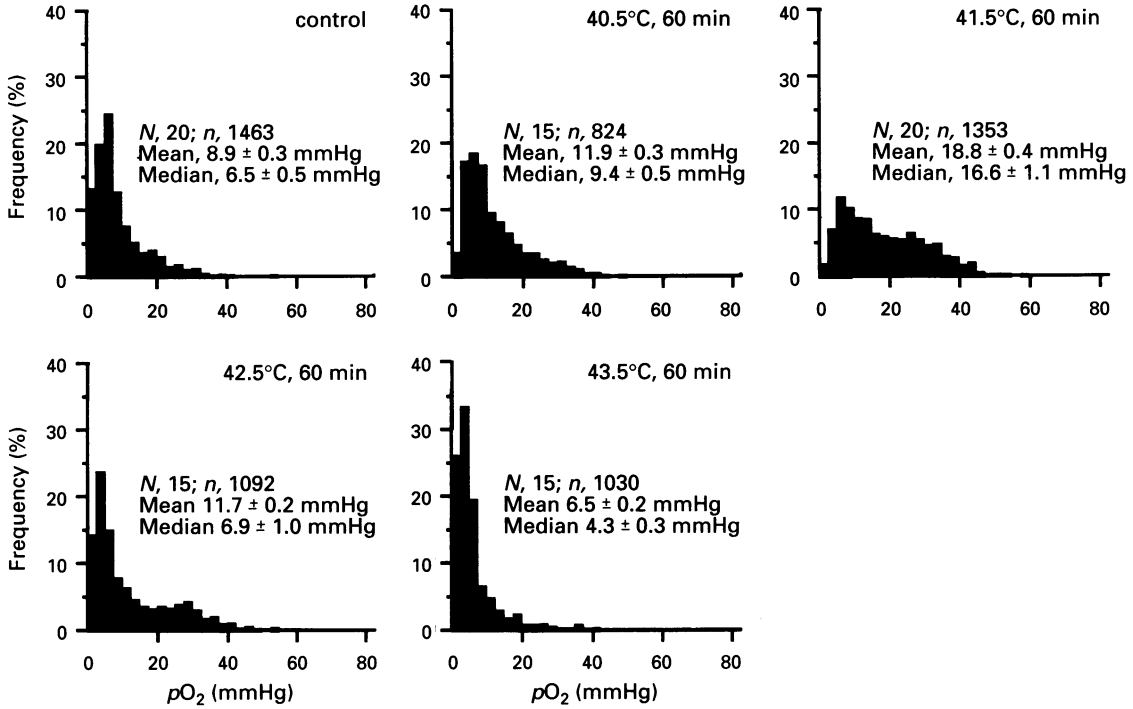

Figure 1 pO_2 histograms of FSaII tumours heated at different temperatures for 1 h. The pO_2 was measured immediately (0 h) after heatings. *N*, number of tumours studied; *n*, number of pO_2 values obtained. Mean and median pO_2 in individual tumours were calculated and the group averages of these values with 1 s.e. are shown.

xylazine at a dose of 87 mg kg^{-1} and 15 mg kg^{-1} respectively. The hairs on the tumour-bearing legs were clipped with an electrical clipper and the rats were placed on a plexiglas board on the top of a water bath. The legs were immersed vertically in a preheated water bath through holes in the board (Lokshina *et al.*, 1985). A thermal insulating pad was placed underneath the rats to minimise the rise in body temperature. The thermometry process for the tumours and water bath was the same as that for the FSaII tumours as described above.

Measurement of tumour pO_2

The pO_2 in FSaII tumours and R3230 AC tumours was measured with an Eppendorf pO_2 histograph (Eppendorf, Hamburg, Germany). The specifications of this device have been previously reported (Kallinowski *et al.*, 1990; Song *et al.*, 1995). The animals were laid on a plexiglas board and the tumour-bearing legs were gently stretched and affixed by taping the foot to the board. The body temperature (rectal temperature) of the animals was maintained close to 37°C by controlling the temperature of the heating pad underneath the board. Self-adhering reference electrodes (SynCor National ECG Electrode) were attached to the shaved dorsal surface of the animals. A pO_2 electrode (300 μm diameter, Eppendorf) was inserted manually into the tumours through small incisions made in the skin over the distal side of the tumour. The electrode was then advanced by a computer-controlled system measuring pO_2 along the track: the electrode was advanced by 0.7 mm forward steps, immediately withdrawn by 0.3 mm to reduce the compression pressure and the pO_2 was measured. The same procedures were repeated, resulting in a 0.4 mm net forward advancement of the electrodes after each pO_2 measurement. In FSaII tumours, the pO_2 was measured along three horizontal and parallel tracks in a lower layer and two parallel tracks in an upper layer in each tumour. In the R3230 AC tumours, the pO_2 was measured along 8–10 parallel tracks. After measurement of pO_2, the tumour temperature was measured using a needle-type thermocouple and the measured pO_2 values were corrected for the temperature in the central part of the tumours which was 34°–36°C. The mean and median pO_2 as well as the relative frequency (%) of pO_2 readings lower than 5 mmHg in each tumour were calculated, and the averages of these pO_2 parameters for each experimental group was obtained. The statistical significance of the difference between the pO_2 in control tumours and that of the experimental groups was analysed using the non-parametric Mann–Whitney *U*-test when the sample numbers of either of the comparing groups was smaller than 20 and using the Wilcoxon rank sum test when the sample numbers exceeded 20 in either of the comparing groups.

Results

FSaII tumours

pO_2 in FSaII tumours measured immediately after heating at different temperatures for 1 h are shown in Figure 1. In the control FSaII tumours, the mean ($\pm$1s.e.) pO_2 and the median pO_2 were 8.9 ± 0.3 mmHg and 6.5 ± 0.5 mmHg respectively. Heating for 1 h at 40.5°C and 41.5°C increased the tumour pO_2. The median pO_2 immediately after heating the tumours for 1 h at 41.5°C was 16.6 ± 1.1 mmHg, which was about 2.5 times higher than that in the control tumours ($P < 0.001$). The pO_2 in tumours heated for 1 h at 42.5°C was similar to that in the control while the pO_2 in tumours heated for 1 h at 43.5°C was markedly lower than the pO_2 in the control tumours ($P < 0.001$). Since the largest increase in tumour pO_2 occurred upon heating at 41.5°C for 1 h, we investigated in detail the kinetics and magnitude of changes in pO_2 during and after heating at 41.5°C for 1 h. Figure 2 shows that the tumour pO_2 remained essentially unchanged during the first 15 min heating at 41.5°C, and then progressively increased until the end of 1 h heating. After the heating, the tumour pO_2 declined, but it was still significantly higher than that in the control tumours at 24 h ($P < 0.05$) after heating. At 48 h after heating, the mean and median pO_2 were almost the same as those in the control tumours (data not shown). The % frequency of pO_2 readings lower than 5 mmHg in FSaII tumours heated for 1 h at

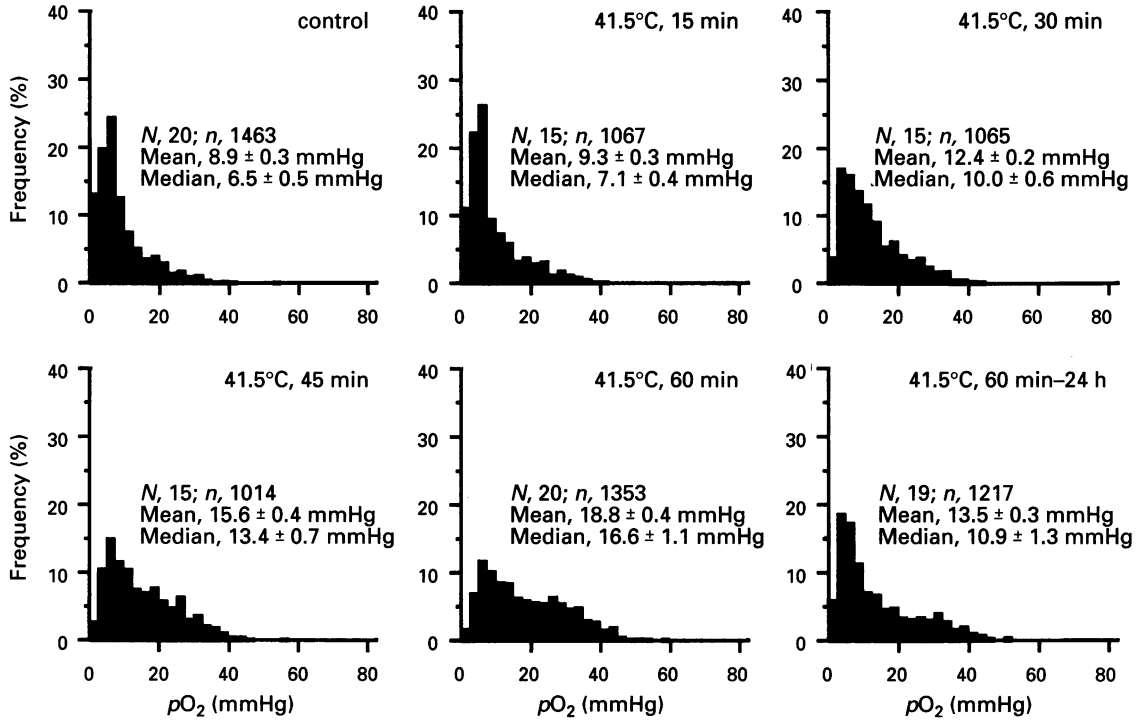

Figure 2 pO_2 histograms of FSaII tumours measured immediately (0 h) after heating at 41.5°C for 15 min–1 h. Histogram of pO_2 measured 24 h after heating at 41.5°C for 1 h is also shown. *N*, number of tumours studied; *n*, number of pO_2 values obtained. Mean and median pO_2 in individual tumours were calculated and the group averages of these values with 1 s.e. are shown.

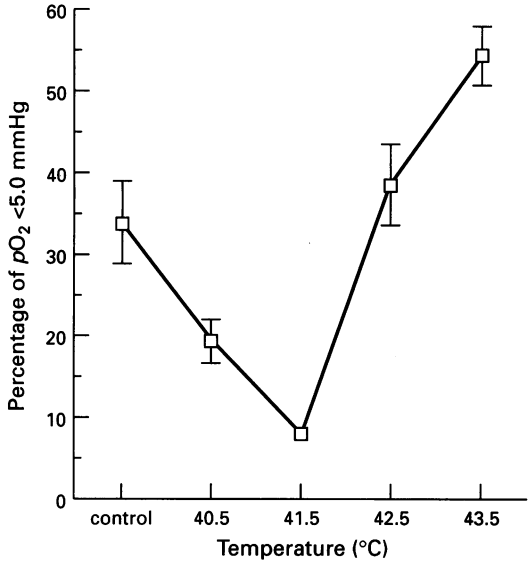

Figure 3 Relative frequency (%) of pO_2 readings lower than 5.0 mmHg in FSaII tumours heated for 1 h at different temperatures. Each data point shown is the average of 15–20 tumours and the bars are 1 s.e.

different temperatures are shown in Figure 3. In the control FSaII tumours, 33.8±5.2% of pO_2 readings were lower than 5.0 mmHg. On the other hand, only 7.9±1.4% of pO_2 readings were lower than 5.0 mmHg when tumours were heated at 41.5°C for 1 h. The frequency of pO_2 readings smaller than 5 mmHg, increased as the heating temperature was raised above 41.5°C. After heating for 1 h at 43.5°C, the frequency of pO_2 readings lower than 5 mmHg was markedly greater than that in the control tumours.

R3230 AC tumours

Figure 4 shows the changes in pO_2 values in R3230 AC tumours upon heating at different temperatures. The mean and median pO_2 in the control tumours were 9.8±0.6 mmHg and 3.7±0.3 mmHg respectively. The mean and median pO_2 increased significantly when the tumours were heated at 41.5°C for 30–60 min ($P<0.05$). Heating at 42.5°C for 30 min increased the mean pO_2 and median pO_2 to 20.2±1.4 mmHg and 12.2±1.8 mmHg respectively, which were about two times and three times those in the control tumours ($P<0.05$). However, the pO_2 in tumours heated at 42.5°C for 60 min was lower than that in the tumours heated for 30 min and was similar to the control tumour pO_2 level. The pO_2 in R3230 AC tumours measured 24 h after heating at 42.5°C for 30 min was slightly higher than that in the control tumours although the increases were not statistically significant. The % frequency of pO_2 readings lower than 5 mmHg in control R3230 AC tumours was 62.2±2.1% and it decreased as the tumours were heated at 40.5–42.5°C for 30 min (Figure 5). In the tumours heated for 30 min at 42.5°C, about 37.1±2.9% of the pO_2 readings were lower than 5 mmHg. In the tumours heated at 40.5–42.5°C for 30 min, the percentage of pO_2 less than 5 mmHg were significantly lower than those in control tumours ($P<0.05$).

Discussion

We have shown that modest temperature hyperthermia (MTH) causes a significant increase in pO_2 in FSaII tumours in C3H mice and R3230 AC tumours in Fischer rats. The increase in tumour oxygenation may result from an increase in tumour blood flow and/or a decline in oxygen consumption by the tumour cells. It has been reported that oxygen consumption by tumour cells is increased by hyperthermia at modest temperatures (Vaupel, 1990). On the other hand, heating at modest temperatures, i.e. 40–42°C, has been reported to increase blood flow in rodent tumours although heating at higher temperatures decreases tumour blood flow (Dewhirst *et al.*, 1984; Jain and Ward-Hartley, 1984; Reinhold and Endrich, 1986; Song, 1984; Vaupel, 1990). We have recently reported that in R3230 AC tumours of rats, the blood flow increased significantly upon heating at 42.5°C for 15–60 min (Nah *et al.*, 1996). It would therefore be reasonable to assume that the increase in tumour

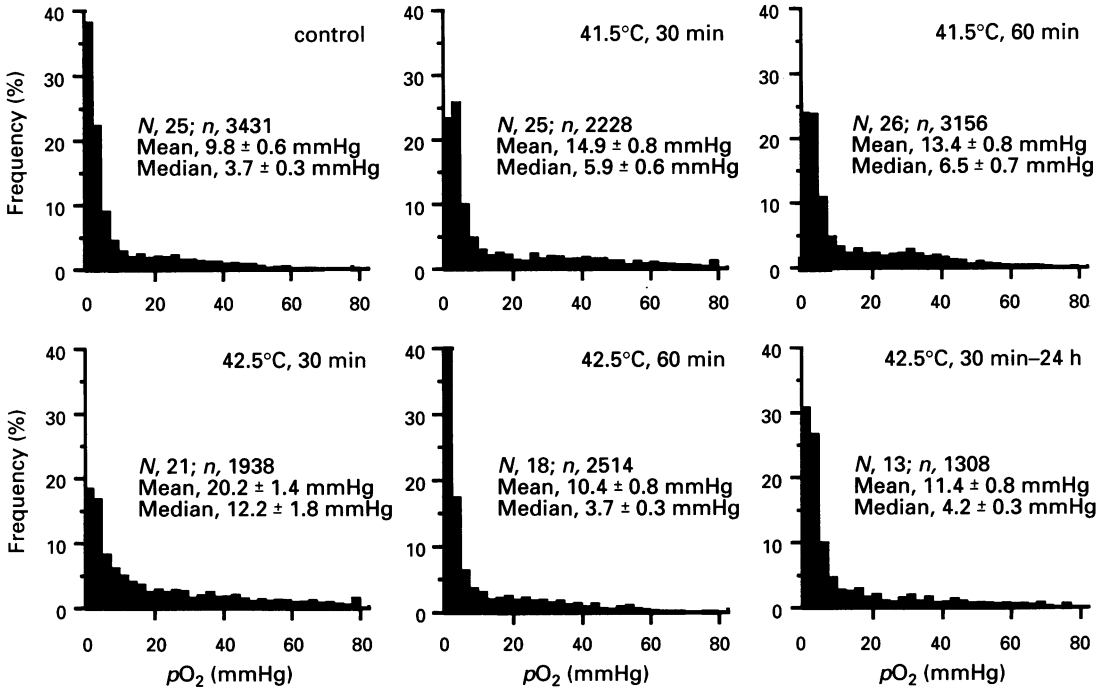

Figure 4 pO_2 histograms of R3230 AC tumours heated for 30 min or 60 min at 41.5°C or 42.5°C. The pO_2 was measured immediately or 24 h after the heatings. *N*, number of tumours studied; *n*, number of pO_2 values obtained. Mean and median pO_2 in individual tumours were calculated and the group averages of these values with 1 s.e. are shown.

oxygenation in the tumours which received MTH, as observed in the present study, was not caused by a decrease in oxygen consumption but was caused mainly by an increase in tumour blood flow. One might wonder how the newly formed tumour vasculatures, which are devoid of smooth muscle, are able to respond to heating to cause an increase in blood flow. It should be noted that a considerable part of the vascular bed in tumours is comprised of normal tissue blood vessels which are incorporated into the growing tumour mass. When heated at modest temperatures, the normal blood vessels, including arteries, in the tumours might dilate and thus blood flow as well as pO_2 in the tumours would increase.

It is not surprising that the magnitude and kinetics of changes in tumour pO_2 caused by heating are dependent on the temperatures and duration of heating. As shown in Figure 2, the pO_2 in FSaII tumours increased progressively during 1 h heating at 41.5°C. Heating for 1 h at temperatures higher than 41.5°C, i.e. 42.5°C and 43.5°C, either failed to increase or even reduced the pO_2 in FSaII tumours (Figure 1). It is reasonable to assume that the newly formed vasculatures in FSaII tumours are damaged and thus the tumour pO_2 is decreased upon heating at temperatures higher than 41.5°C. In R3230 AC tumours, heating at 42.5°C for 30 min was most effective at increasing tumour pO_2 when compared with heating at 40.5°C or 41.5°C for 30 min (Figure 4). When heating at 42.5°C lasted longer than 30 min, however, the tumour pO_2 began to decrease returning to the control level by the end of 1 h heating.

It is important to note that the increases in pO_2 in heated tumours resulted in a significant reduction in the frequency of pO_2 readings lower than 5 mmHg both in FSaII tumours (Figure 3) and R3230 AC tumours (Figure 5) indicating that significant fractions of radiobiologically hypoxic cells in the tumours are reoxygenated by MTH. Our preliminary studies, using the paired cell survival curve method, clearly indicated that MTH significantly reduces the hypoxic cell fraction in FSaII tumours (data not shown). Another important and clinically relevant observation we made is that pO_2 remained elevated for 24 h after heating at 41.5°C for 60 min in FSaII tumours (Figure 2). These results suggest that the indirect radiosensitisation of tumours by MTH may last as long as 24 h.

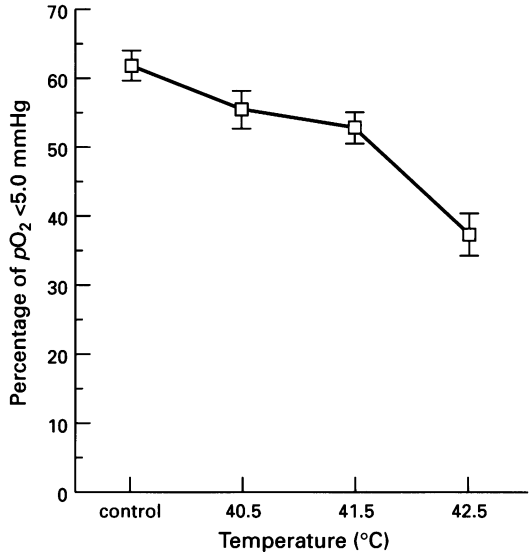

Figure 5 Relative frequency (%) of pO_2 readings lower than 5.0 mmHg in R3230 AC tumours heated for 30 min at different temperatures. Each data point shown is the average of 20–25 tumours and the bars are 1 s.e.

Our observations that MTH increases tumour oxygenation are in agreement with the following reports by other investigators. Vaupel *et al.* (1982) reported that the oxygenation of DS-carcinoma in rats increased when the tumours were heated at 40°C, but it decreased when the heating temperature was raised to 43°C. Hetzel *et al.* (1992) observed that the pO_2 in a mammary adenocarcinoma grown in the flank of C3H mice increased 24–48 h after an exposure of the tumours to modest heat doses which could cure 10% of the treated tumours. Whether pO_2 in human tumours is also increased by MTH has not yet been determined. The temperature of s.c. grown FSaII tumours and R3230 AC tumours in unanaesthetised animals in the present study was 34–35°C while human tumour tempera-

tures are probably close to 37°C. Therefore, the increase in pO_2 caused by heating at 41–42°C in human tumours might be less than that in rodent tumours. Nevertheless, it is reasonable to expect that the pO_2 in human tumours would be increased significantly by MTH, as Oleson suggested (Oleson, 1995).

In conclusion, MTH is potentially effective in causing indirect radiosensitisation of tumours through an improvement of tumour oxygenation. In view of the fact that human tumour temperature can easily be raised to 40–42°C using currently available clinical hyperthermia devices, the usefulness of hyperthermia at modest temperatures as an indirect radiosensitiser of human tumours through increasing tumour oxygenation warrants further investigation.

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