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Histologic analysis of liver tissue following hepatic arterial infusion of ferromagnetic particles in a rabbit tumour model

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Abstract

It is possible to arterially embolize liver tumours in small animal models with ferromagnetic particles that generate hysteretic heating on exposure to an alternating magnetic field. The aim of this study was to determine the response of hepatic tissue to arterial infusion of ferromagnetic particles. Eight rabbits containing hepatic VX2 carcinomas received a hepatic arterial infusion of ferromagnetic particles suspended in lipiodol. Four rabbits were sacrificed after 60 min to determine the acute tissue response, and the other four rabbits were sacrificed after 14 days to determine the longer-term tissue response. The tumour, normal hepatic parenchyma (NHP), lungs and gallbladder of each subject were examined using light microscopy, and chemically analysed for iron concentration. Large aggregates of particles embolized within the tumour vasculature. There was only a sparse distribution of particles in the NHP, with no acute tissue response. The tumour to NHP iron concentration ratio was 4.9. Particles also lodged in blood vessels of the gallbladder wall. Two weeks after infusion there were isolated foci of necrosis in the NHP, and macrophages were associated with particle aggregates, which were also observed within multinucleated giant cells. There was no evidence that particles embolized in the lungs. Hepatic arterial infusion of ferromagnetic particles suspended in lipiodol resulted in excellent tumour targeting with no acute tissue reaction in the NHP, and no evidence of pulmonary embolization. After 14 days there was evidence of phagocytosis of the particles in NHP, but not in the tumour tissue. However, the suspension caused multiple foci of infarction in NHP, probably due to occlusion of larger arteries.

Introduction

It is well established that liver tumours larger than 1mm in size derive virtually all their blood supply from the hepatic arterial system, while normal hepatic parenchyma (NHP) receives most of its blood supply from the portal venous system (Breedis *et al.* 1954; Stribley *et al.* 1982; Archer *et al.* 1989, 1990). This differential in blood supply between hepatic tumours and NHP has been successfully exploited in treatment modalities such as Hepatic Arterial Chemotherapy (HAC), Selective Internal Radiotherapy (SIRT) and Transcatheter Arterial Chemoembolization (TACE), which target liver tumours with chemotherapeutic agents and radiation (Burton *et al.* 1989; Ho *et al.* 1998; Kemeny *et al.* 1999; Campbell *et al.* 2000). This same principle can be used to target liver tumours with ferromagnetic particles, and therefore high temperatures on exposure to an alternating magnetic field. Arterial Embolization Hyperthermia (AEH) is an experimental modality of hyperthermia that consists of the selective arterial embolization of liver tumours with ferromagnetic particles, followed by exposure to an alternating magnetic field to generate hysteretic

heating of the embolized particles, and hence the surrounding tissue. Recent studies have reported that it is possible to effectively target liver tumours in small animal models with hyperthermia using this technique (Mitsumori *et al.* 1996; Minamimura *et al.* 2000; Moroz *et al.* 2001a, b; Jones *et al.* 2001), and two of these studies have reported significant tumour responses (Minamimura *et al.* 2000; Jones *et al.* 2001).

Although more detail on the technique of AEH is now emerging in terms of the type of ferromagnetic particles used, magnetic field generation, heating rates and tumour growth responses, several important issues have yet to be addressed. The recent studies listed above are lacking in detail on the microscopic distribution of particulate iron oxide in liver tumours and NHP, and they give no information regarding the acute and longer-term tissue reactions to these particles. It is also unknown if the particles can pass through the hepatic circulation and into the lungs. Furthermore, it is not known if the embolized particles are cleared from hepatic tissue, or whether they form permanent inert deposits. The aim of this study is to address these issues with a view to evaluating the safety of AEH.

Method

Ethics approval

All work in this study was performed with approval from the Royal Perth Hospital Animal Ethics Committee (Approval 13/97), in accordance with the Statement on Animal Experimentation by the Australian National Health and Medical Research Council.

Subjects

This experimental study was performed using 16 to 20 week old New Zealand half lop rabbits weighing three to four kilograms. At day zero, under aseptic conditions and a barbiturate general anaesthetic, the livers of eight rabbits were implanted with a 1 mm piece of tumour from the established VX2 carcinoma line. This piece of tumour was taken fresh from the hind limb of a passage rabbit and implanted into the centre of the left medial liver lobe (the tumour lobe) (Rowett *et al.* 1975), with hepatic access gained via a 5 cm midline upper abdominal incision. After closure of the abdominal wound, each rabbit was allowed to recover from the anaesthetic and the tumour was allowed to grow for 14 days.

Ferromagnetic suspension

Magnetic iron oxide particles (γ-Fe_2O_3) of approximately 150 nm in diameter were suspended in lipiodol (50 mg of particles in 1 ml of lipiodol) by ultrasonication for five minutes followed by agitation on a vortex mixer for one minute.

Embolization technique

At day 14, the eight rabbits were subjected to a barbiturate induction general anaesthetic, which was maintained with halothane. A 10 cm midline abdominal incision commencing just inferior to the xiphoid process, with peripheral retraction of the abdominal wall, provided exposure of the upper abdominal viscera. With the hepatic artery clamped, the cystic artery was catheterised up to its junction with the hepatic artery with a single lumen polyethylene tube (od=0.8 mm, id=0.5 mm) attached to a 1 ml syringe. The ferromagnetic suspension was heated to 40 °C in a water bath prior to infusion to improve the flow through the catheter. After agitation on a vortex mixer, the suspension was steadily infused via two 0.5 ml aliquots with a heparinised saline flush of 0.25 ml immediately after each aliquot. Hepatic arterial flow was restored for two minutes after each 0.25 ml flush. After the suspension had been infused the catheter was removed, the cystic artery ligated and the hepatic artery unclamped to restore hepatic arterial blood flow.

To determine the acute tissue effects of the hepatic arterial infusion, four rabbits were then maintained under anaesthesia for 60 min to allow settling of the infused particles, before sacrifice via a barbiturate overdose.

To determine the longer-term tissue effects, the other four rabbits had their laparotomy repaired and were then allowed to survive for a further 14 days before sacrifice via a barbiturate overdose.

Histological analysis for iron particle distribution and tissue response

After each subject was sacrificed, the liver and lungs were harvested for histologic and chemical analysis. Two to six specimens were obtained from the liver tumour, adjacent NHP, lungs and gallbladder of each subject. The specimens were fixed and then processed in paraffin, mounted onto slides and stained with haematoxilin and eosin. Each specimen was examined for particle distribution and tissue reaction using light microscopy.

Chemical analysis for iron particle distribution

After the samples for histologic analysis had been obtained, the tumour, the liver lobe containing the tumour (adjacent NHP) and the lungs were weighed and digested in acid. The resulting solutions were analysed for iron content by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (Willard 1988). This enabled the average iron concentration in the tumour, adjacent NHP and lungs to be calculated in milligrams of iron per gram of tissue.

The livers and lungs of five further rabbits that did not receive a hepatic arterial infusion of ferromagnetic iron particles were digested and chemically analysed for baseline iron content. Intrinsic hepatic and pulmonary iron is in cationic form bound to large anionic proteins within cells or in blood (ferritin or haem, respectively). As this iron will be detected in chemical analysis of hepatic and pulmonary tissue, it will lead to an overstatement of the actual tissue iron due to the hepatic arterial infusion. This baseline iron concentration must therefore be subtracted from tissue iron concentrations obtained by chemical analysis. The baseline hepatic iron concentration was determined by ICP-AES analysis of 56 NHP samples obtained from the five tissue control rabbits that did not receive an iron particle infusion. The mean iron concentration in this group of samples was 0.24 mg/g (standard deviation = 0.03). Therefore 0.24 mg/g was subtracted from each hepatic tissue iron concentration obtained from ICP-AES.

The baseline pulmonary iron concentration from 20 samples obtained from the same five rabbits was 0.16 mg/g (standard deviation = 0.08). To determine if the iron concentration in lung tissue was significantly above baseline, each pulmonary iron concentration obtained via ICP-AES (both lungs from each subject were digested, resulting in 16 samples) was compared with the 95% confidence limit about the mean baseline pulmonary iron concentration (i.e., 0.12, 0.20). If the iron concentration was within this range, it was concluded that it was not significantly different from the baseline pulmonary iron concentration. The Mann-Whitney U-Test was also used to test if the average iron concentration in the lungs of the subjects that received a hepatic arterial infusion was significantly different to that of the subjects that did not.

Table 1. Tissue iron concentrations following hepatic arterial infusion. Subjects 1 to 4 were sacrificed 60 min after arterial infusion, while subjects 5 to 8 were sacrificed 14 days after arterial infusion.

Subject	Tumour iron (mg/g)	NHP iron (mg/g)	T:N
1	1.55	0.35	4.4
2	3.33	0.59	5.6
3	2.23	0.32	7.0
4	2.32	0.56	4.1
5	2.59	0.52	5.0
6	1.39	0.16	8.7
7	2.40	0.60	4.0
8	2.81	0.59	4.8
Median	2.36	0.54	4.9

Results

Chemical analysis for iron

Table 1 shows the iron concentrations obtained in tumour tissue and NHP, both in the four subjects that were sacrificed 60 min after arterial infusion (subjects 1 to 4), and in the four subjects that were allowed to survive to 14 days (subjects 5 to 8). The table demonstrates that the arterial infusion resulted in a high degree of tumour targeting, with a median tumour to normal (T:N) iron concentration ratio of 4.9.

The median pulmonary iron concentration in the 8 subjects (16 lungs) was 0.14 mg/g (min = 0.10 mg/g, max = 0.19 mg/g), which was not statistically different from the baseline iron concentration, $P = 0.65$. Furthermore, comparison of each of the 16 pulmonary iron concentrations with the 95% confidence limit about the mean baseline iron concentration showed that 15 iron concentrations were within the limit, and one concentration (10 mg/g) was just below the lower limit. These findings suggest that the infused iron particles did not exit the liver and lodge in the lungs, acutely or over the two week follow up period.

Histological analysis sixty minutes after infusion

Examination of the NHP revealed a very sparse distribution of iron particles, with only occasional portal tracts containing particles (Figure 1). The particles tended to form aggregates and were confined within vessels of portal tracts (Figure 2). The architecture of the NHP was normal with no signs of inflammation, congestion or cell injury (Figures 1 & 2).

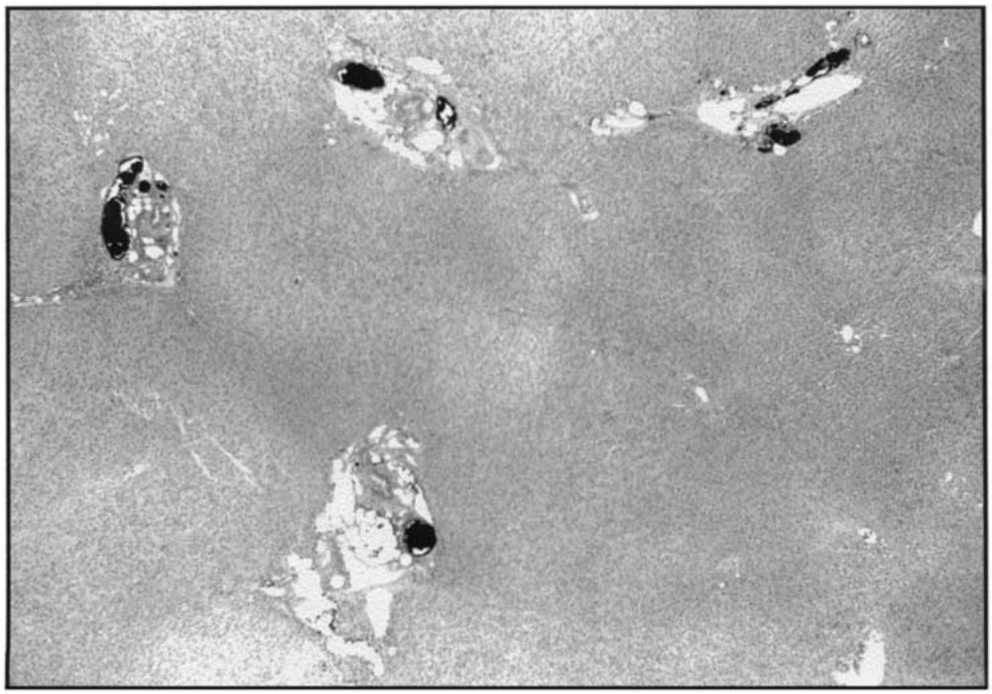

Fig. 1. Aggregates of ferromagnetic particles within portal tracts in the NHP. (Magnification 72X).

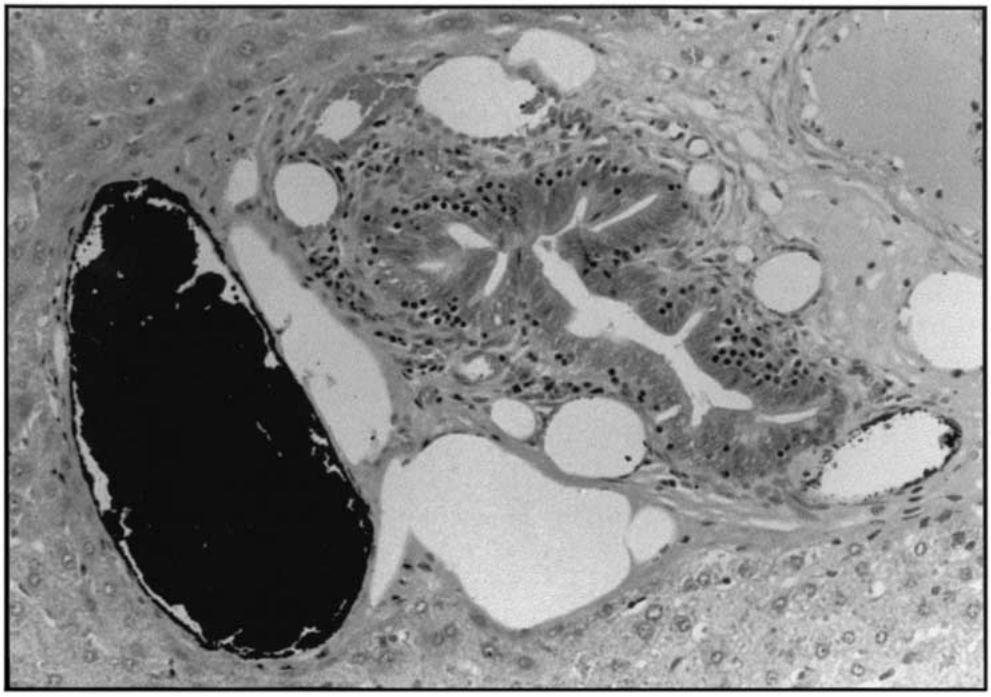

Fig. 2. Particle aggregates confined within a blood vessel adjacent to a bile duct of a portal tract in NHP. (Magnification 796X).

Large deposits of aggregated particles were seen within dilated blood vessels and vascular channels throughout the tumour (Figure 3). Little or no extravasation of particles was seen. The adjacent NHP showed sinusoidal congestion, triaditis and moderate amounts of particle aggregates in the vessels of portal tracts. These findings in the tumour and NHP support the results of the chemical tissue analysis i.e., there was a greater concentration of iron particles within the tumour tissue as compared to the NHP.

A section of the gallbladder showed iron particles in arteries in the wall due to communication with the hepatic arterial system (Figure 4). Examination of the lungs failed to show evidence of embolized particles, suggesting that the particles did not pass through the liver and lodge in the lungs.

Histologic analysis 14 days after infusion

After 14 days there were between 2 to 6 six foci of necrosis (0.5 to 2 cm in diameter) surrounded by inflammation, fibrosis and calcification in the liver parenchyma in each subject. Only some of these necrotic areas contained iron particles. There was no recanalization of blood vessels. Atrophy of hepatic plates was present adjacent to the infarcts (Figure 5). Most of the iron particles in NHP were confined within blood vessels, but small amounts were seen in the soft tissue adjacent to vessels, and in the sinusoids. Macrophages were associated with occasional particle aggregates (Figure 6), and particle aggregates were also seen within multinucleated giant cells (Figure 7), indicating that phagocytosis of the foreign particles had begun by day 14.

The tumour also contained iron particles within vessels, but there was no tumour necrosis or reaction to the particles. This suggests that the presence of the particles alone would have little direct effect on tumour growth. In contrast to the NHP 14 days after infusion, there was no evidence of phagocytosis of the particles in the tumour tissue.

Again, examination of pulmonary tissue failed to show evidence that the particles had passed through the liver and lodged within the lungs.

Discussion

Hepatic arterial infusion of 150 nm γ-Fe_2O_3 particles suspended in lipiodol resulted in excellent targeting of liver tumours (median T:N = 4.9), and hence the potential for tumour targeting with hyperthermia on the subsequent exposure to an alternating magnetic field. Only small aggregates of intravascular particles were demonstrated in occasional portal tracts of the NHP, with no evidence of acute tissue injury or congestion in the vast majority of NHP. However, large amounts of aggregated particles embolized within the vasculature of the liver tumours, which mirrored the tissue concentrations of iron determined by chemical analysis. The major reason for this targeting is the arterial origin of tumour blood vessels, their architecture, and the fact that NHP is largely supplied by the portal venous system. Tumour blood vessels are often dilated, tortuous and saccular with excessive branching and blind endings (Vaupel 2000). The blood vessels of tumours also lack innervation and smooth muscle, which leads to a lack of regulatory control. Furthermore, these blood vessels often have incomplete basement membranes and missing endothelial cells which leads to increased vascular permeability in the tumour (Hoogewoud 1993). Venous drainage of tumour tissue is often poor and incomplete. These features of tumour vasculature are therefore likely to lead to physical sludging and entrapment of viscous lipiodol containing ferromagnetic particles, as demonstrated in Figure 3.

On the application of an alternating magnetic field, the occasional aggregates of particles in NHP (Figure 1) would generate heat by hysteresis loss effects. Each aggregate would act as a point source of heat, which would be conducted into surrounding tissue and blood. The sparse distribution of particles in the NHP suggests that the large portal blood flow would cool these point sources of heat and thus prevent thermal damage to NHP. On the other hand, the much higher concentration of particles in the tumour (Figure 3 and Table 1) would lead to the generation of far more heat in the tumour tissue, which together with the poorer cooling within tumours (due to the absence of an ordered, high flow, portal venous system) would result in the attainment of greater temperatures in liver tumours as compared to NHP.

Examination of gallbladder tissue showed some large deposits of intravascular aggregates of particles that resulted, presumably, from communication with the hepatic arterial system. Exposure to an alternating magnetic field would therefore be likely to cause necrosis of the gallbladder wall. In a clinical setting a prophylactic cholecystectomy should be performed before arterial embolization, to prevent this potentially serious complication. The extrahepatic biliary system

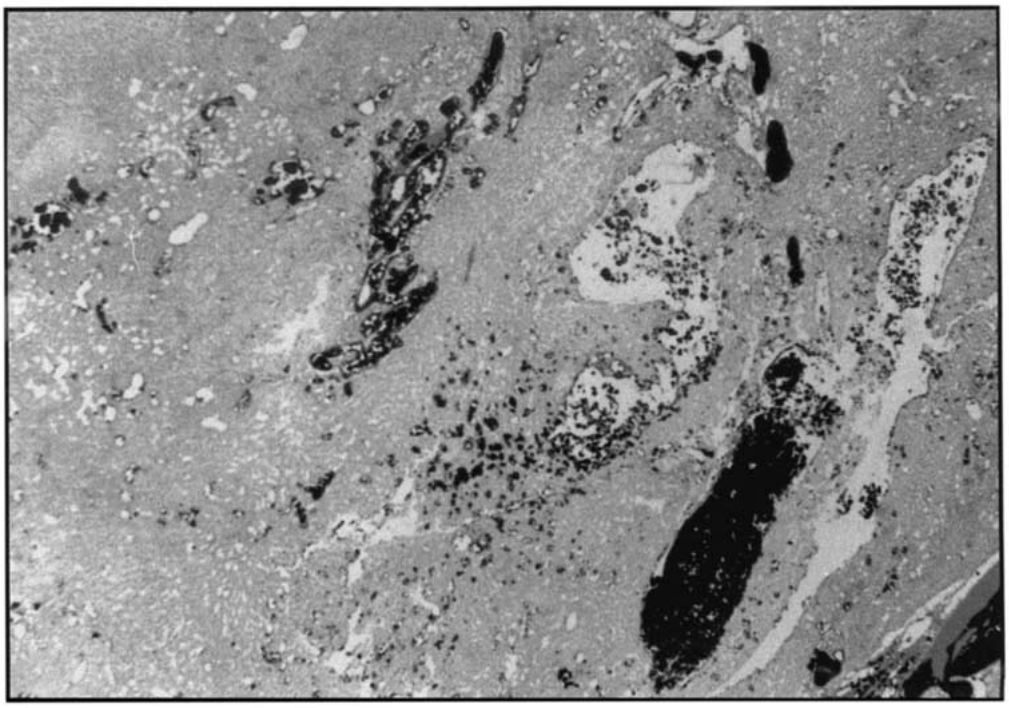

Fig. 3. Aggregates of ferromagnetic particles that have embolized within the disordered and chaotic vascular system of a tumour. (Magnification 76X).

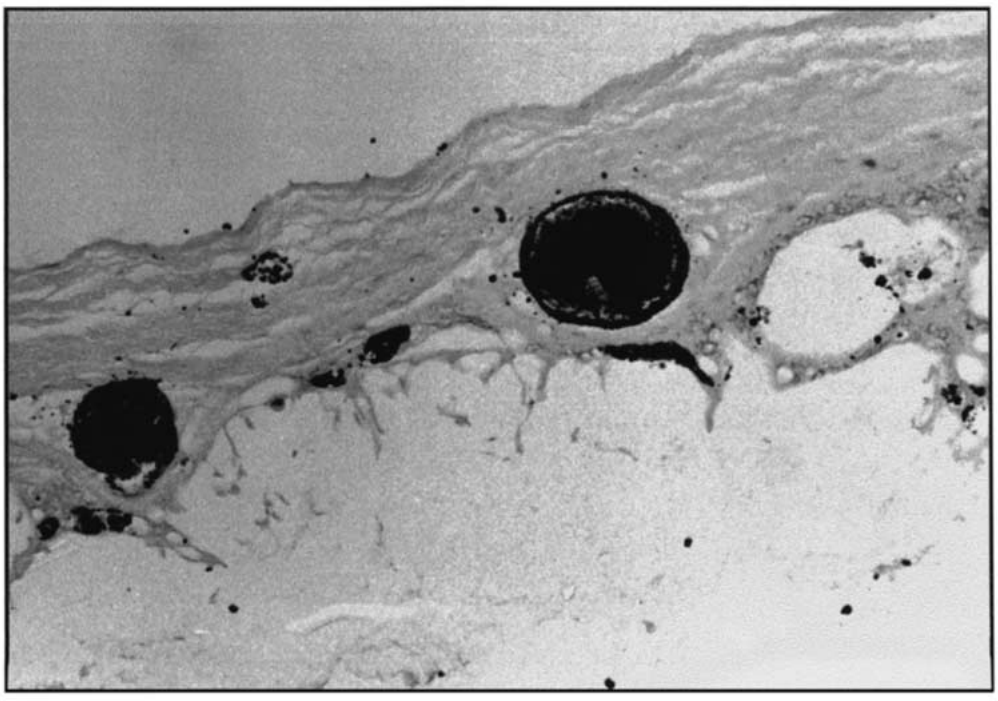

Fig. 4. Particle aggregates within the gallbladder wall. (Magnification 72X).

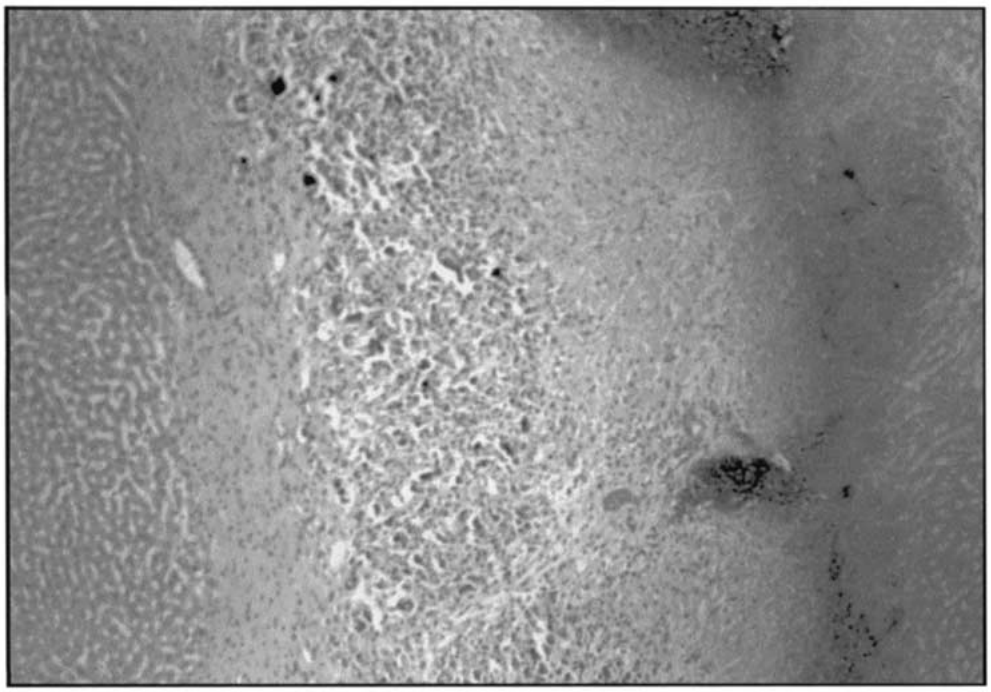

Fig. 5. Section through an area of non-tumourous parenchyma 14 days after infusion of ferromagnetic particles. Necrotic tissue (right) is surrounded by an inflammatory reaction, fibrosis and calcification (centre). To the left is atrophic NHP.

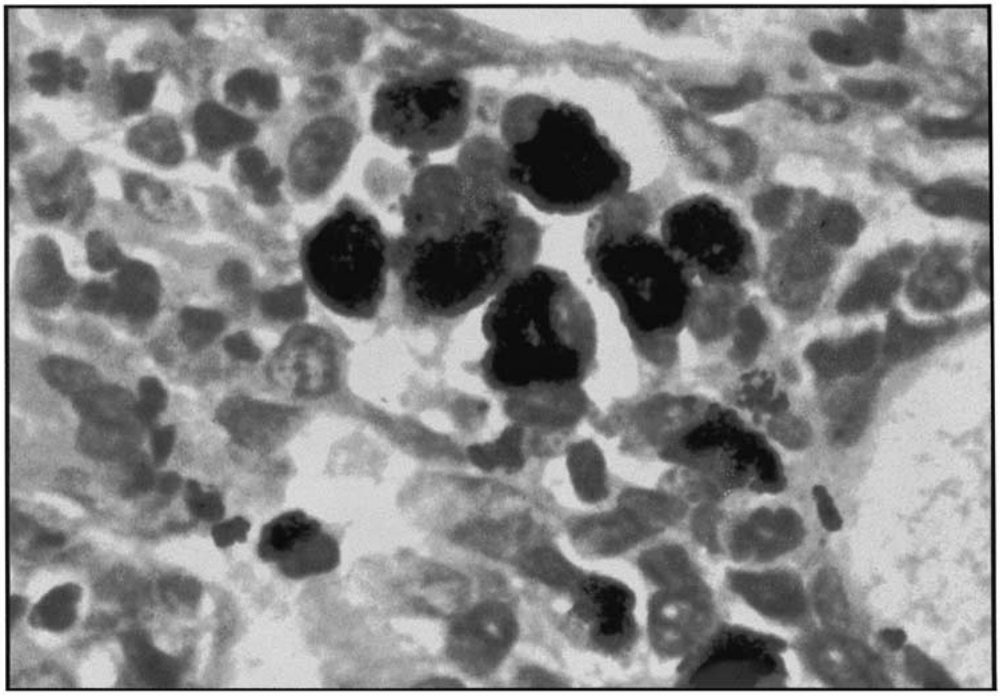

Fig. 6. Macrophages in association with particle aggregates in the NHP 14 days after infusion. (Magnification 894X).

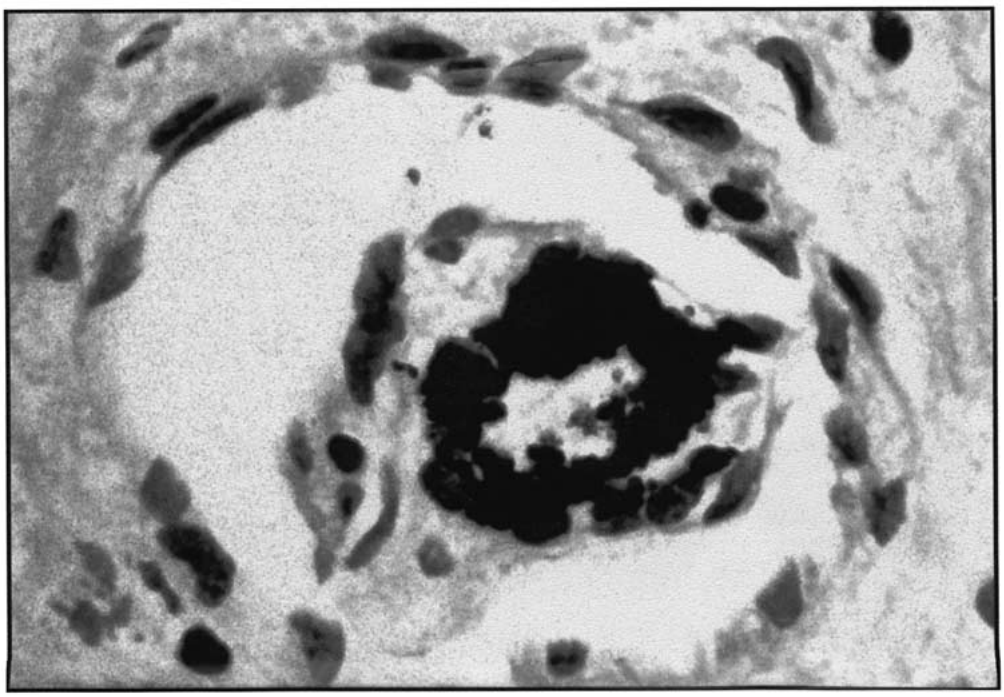

Fig. 7. Particle aggregates that have been engulfed by a multinucleated giant cell in the NHP 14 days after infusion. (Magnification 894X).

also receives a supply from the hepatic artery. It is therefore possible that ferromagnetic particles could lodge in the walls of the hepatic, cystic and bile ducts. The vasa vasorum of the hepatic artery itself could provide a pathway for ferromagnetic particles to lodge in the walls of the artery. These structures could suffer ischaemic damage as a result, and on exposure to an alternating magnetic field they could also suffer thermal damage. In a clinical situation therefore, the hepatic arterial catheter should be inserted well into the liver substance to bypass the arterial blood supply to these structures.

This study also found no evidence that the ferromagnetic particles injected into the hepatic arterial system exited the liver and passed into the lungs. This could have been possible if significant arteriovenous shunting existed in the tumour tissue. The deposition of ferromagnetic particles in the pulmonary vasculature would obviously be highly undesirable due to the risk or infarction and thermal damage, which could lead to significant respiratory impairment. It could be that the tumour model used in this study does not tend to form such shunts, or that the viscosity of our suspension was sufficient to hold the particles within the NHP and disordered vascular system of the tumour. Another factor that may have prevented the lodgement of particles in the lungs was the tendency for the particles to form large intravascular aggregates, as shown in Figures 2 & 3.

Of concern is the finding of infarcted NHP 14 days after infusion. It is likely that this resulted from vascular occlusion by the suspension. Some of the infarcted areas did not contain ferromagnetic particles, which suggests that the vascular occlusion occurred at some distance, with infarction of NHP distal to the obstruction. The finding of atrophic hepatic plates suggests that even more hepatic tissue was subject to a milder level of ischaemia. Our findings are consistent with those of Sako *et al.* (1982). In this latter study, iron microspheres of 10 to 30 um diameter (larger than the particles used in our study) suspended in a viscous polysaccharide were arterially infused into hepatic VX2 carcinomas in rabbits. A similar effect on NHP was reported. In patients with only a relatively small amount of healthy liver tissue, this could result in a poor outcome. Further work in large animals is needed to determine the risk of hepatic ischaemia and the tolerance limits. Less viscous suspensions could also be developed to help avoid vascular occlusion.

The finding of particle aggregates within giant cells and in association with macrophages in the NHP suggests that the particles may be eliminated from the

liver over time. It is also possible that the particles may form inert deposits within phagocytic cells, as they have an inert oxide coating, analogously to pulmonary asbestos bodies (oxide coated silicon) following asbestos exposure. Histologic examination and particle quantification over a longer time period than the one used in this study is needed to determine if the levels of iron in the NHP decrease as a result of phagocytic clearance, or whether they remain stable.

This study has also shown that there was no reaction to, or phagocytosis of, the particles in the tumour tissue 14 days after infusion. The potential significance of this finding is that the persistence of a high tumour concentration of particles would facilitate repeated hyperthermia treatments. Moreover, if the particles were to be cleared from the NHP as suggested above, and not cleared from tumour tissue, then the application of an alternating magnetic field to generate hysteretic heating of the particles could be delayed to allow time for further magnification of the T:N iron concentration ratio which could improve not only the tumour heating, but also the overall safety of the treatment by further sparing the NHP from thermal damage.

Conclusion

Hepatic arterial infusion of ferromagnetic particles suspended in lipiodol resulted in the embolization of large aggregates of particles within the disordered vascular system of tumours. There was only a sparse distribution in the NHP with no acute tissue response. However, 14 days after infusion there were areas of necrosis in the NHP, probably due to vascular occlusion by the suspension. At this time there was also evidence of phagocytosis of the particles in the NHP, but not in the tumour tissue. There was no evidence that the particles exited the liver and passed into the lungs. Further work in a large animal model is needed to determine the safety of hepatic arterial infusion of 150nm ferromagnetic particles suspended in lipiodol.

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