Formulation of a Charcoal Suspension for Intratumor Injection. Part 1: Study of the Nature, Granulometry, and Concentration

L. Bonhomme-Faivre,[1,6] M. C. Mathieu,[2] J. L. Grossiord,[3] P. Depreatere,[4] G. Couarraze,[3] S. Orbach-Arbouys,[1] F. Puisieux,[5] and M. Seiller[4]

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Purpose. We developed a charcoal suspension formulation to be injected intratumorally so that human breast cancers can be tatooed prior to chemotherapy. This deposit is intended to guide the surgeon at the time of the biopsy and resection, especially when the tumor nodule is not visible. The stain should remain in the tumor as long as the patient is on chemotherapy and should be harmless.

Methods. We studied on the effect on the nature of the charcoal, its granulometric profile, and its concentration. We then measured diffusion in vitro, in gel, and in vivo in experimental tumors.

Results. The formulation selected was prepared with a peat charcoal suspension in water for parenteral injections, with 50% of the particles measuring on average between 2 and 5 μm. The finest particles (<2 μm) seem to produce the greatest in vitro diffusion and are more readily phagocyted by macrophages and thus eliminated from the tumor by those cells.

Conclusions. This charcoal suspension has satisfactory formulation characteristics and diffuses the least, be it in vitro or in vivo, mainly due to the granulometric distribution of the suspension.

KEY WORDS: charcoal; suspension; in vitro diffusion; in vivo labelling; mammary tumor.

INTRODUCTION

The extent of surgery for breast tumors is now as limited as possible for cosmetic and psychological reasons. Protocols include adjuvant therapy to reduce the initial tumor volume with irradiation or chemotherapy (1). Localizing the tumor is rendered very difficult at the time of the surgery due to a drastic reduction in its volume. Different stains have been used, as they tend to disappear rapidly through diffusion (2–5), and surgery must intervene soon after their injection. A charcoal preparation was tested in Sweden in 1980 (6). The diffusion of charcoal is limited when injected in situ to label nonpalpable mammary tumors. We assumed that the migration of the charcoal particles injected intratumorally would invariably be dependent on the components of the preparation. Normally, suspensions should possess the three criteria required for any drug, namely: a) quality: the main pharmacotechnical characteristics such as pH, viscosity, and granulometry should be stable with time. Sedimentation, if observed, should be easy to disperse so that a homogeneous suspension is acquired at the time of the assay and of the injection. b) security: the suspension should produce no undesirable effects at the recommended doses. c) efficacy: the intratumor injection should be easy and harmless, and the label should be easy to detect in the tumor and its diffusion limited in the surrounding tissues. A compromise was sought during the formulation study.

Optimizing the formulation was achieved gradually with diffusion assays in vitro, in gel, and in vivo in mouse tumors at each phase.

MATERIAL AND METHODS

Charcoal

Two types of charcoals were studied: pine wood charcoal LSM (CECA-SA, 92 La Défense) and peat charcoal SX4 (Norit, 93, Le Blanc Mesnil). Norit SX4 charcoal is in keeping with US Food Chemicals Codex requirements. Both were steam activated and washed with phosphoric acid. Surface adsorption of charcoal is of 1000 m^2/g for wood charcoal, and 650 m^2/g for peat charcoal. Both contain 10% water.

Gels

The gel used was prepared to mimic the physiological and chemical characteristics of breast tumors. Human breast tumors are mainly composed of collagen, elastin fibers, and tumor cells. Their water content varies between 80% and 85%, and conductivity from 7.0 to 9.3 milliSiemens/cm (7) for a frequency of 100 megaHertz. An increase in conductivity between tumor cells would be due to a greater membrane permeability.

The pH of human breast cancer is 7.29 ± 0.05 whereas the subcutaneous pH is 7.63 ± 0.03 (8). The gel was prepared according to the El Akoum modified formula (9) based on the use of Eudispert, a polymethylmetacrylate polymer with methyl lipophilic groups and carboxyl and ester hydrophilic groups (Eudispert™; Röhm Pharma).

Animals

C3H female mice, aged 6–8 weeks, were bred at the Institut Gustave Roussy animal experimentation department. The studies were carried out on animals weighing 20–25 g, 21 days after implantating tumorous cells of C3H mouse mammary adenocarcinoma into the hind leg. This is a syngenetic implantable tumor obtained from solid tissue transplants. A 0.5 ml volume of filtered tumoral cell (5×10^5 cells) suspension was injected subcutaneously.

Preparation of the Different Granulometries

Wet Crushing

Charcoal was dispersed in suspension medium and crushed in a planetary ball mill in 20 ml zirconium oxide jars with 12

[1] Laboratoire de Pharmacologie, Département de Pharmacie, Hôpital Paul-Brousse, 14, avenue Paul Vaillant Couturier-94800-Villejuif, France.

[2] Service d'Anatomopathologie, Institut Gustave-Roussy, rue Camilles Desmoulins, 94800-Villejuif, France.

[3] Laboratoire de Physique, Faculté de Pharmacie, rue Jean-Baptiste-Clément, 92290 Châtenay-Malabry, France.

[4] Laboratoire de Galénique, Faculté de Pharmacie, 1, rue Vaubenard, 14000 Caen, France.

[5] Laboratoire de Galénique, Faculté de Pharmacie, rue Jean-Baptiste-Clément, 92290, Chatenay-Malabry, France.

[6] To whom correspondence should be addressed.

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mm diameter balls from the same material. Jars were filled to two-thirds of their capacity. Crushing lasted 20 min.

Micronized Dry Crushing

Charcoal was crushed in a stainless steel microniser (Jet O'Mizer) with a compressed filtered air jet (7 bars).

Preparation of the Suspensions

An appropriate quantity of charcoal was added to water for parenteral injections. Dispersion was obtained in a turbine mixer at a stirring rate of 200 rpm for 10 min at room temperature. The preparation was distributed in penicillin-type bottles, sealed, and sterilized at 120°C during a period of 20 min.

Gel Preparation and Controls

5 g EudispertND were dissolved in 30 mL water for parenteral injection in a beaker. 1.2 to 1.6 g NaOH were dissolved separately in 30 mL water. The latter solution was slowly added to the first one with continuous stirring. A gel is obtained after 8 h at room temperature. 15 g polyethylene glycol 4000 are then dissolved in 40 mL water, or in 30 mL water with 10 mL glycerol, and added to the previous preparation. An 120 mL preparation is obtained and poured into square crystal polystyrene Petri dishes.

pH was measured with a pH-meter analyser multiparameter P407 MCNS 11, calibrated between each measurement with pH 4 and pH 7 buffers.

Conductivity was measured at 20°C at a frequency of 50 Hz with an analyser P 407 MCNS 11 equipped with a conductivity cell YSI 3417 (Bioblock). A second study of conductivity was performed at frequencies ranging from 10 to 500 MHz with the Roussy technique (11).

Such a study was done after selecting the gel composition, the conductivity of which was close to that of the human breast tumors, observed at 100 MHz. The study of rheology was done with a CSL 100 rheometer with a shear stress rate given by a geometry cone/plate on a sample of 3 mL gel. Follow up was done by recording the speed variant D (s-1) according to the shear stress rate (Nm-2). The assay included three phases: a 2 mn phase during which the shear increases, a 2 mn plateau, and a 2 mn phase during which the shear decreases.

Injection into the Animal

The tumor reached a size of 1 to 2 cm in diameter 3 weeks after the injection, and 100 or 50 μL of the charcoal suspension were injected intratumorally at that time. The animals underwent an autopsy 10 days later.

DESCRIPTION OF THE ASSAYS

Suspensions

Granulometry

Measurements were done in a Coulter Counter (Model TA 11, Coultronics, SA 95 Andilly, France).

Sedimentation and Resuspension

50 mL suspension was placed in a 50 mL graduated test tube. Spontaneous sedimentation was measured at room temperature at 5, 10, 15, 20, 30, 40, 60, and 120 min then at 24 and 48 h, at 1 week, and at 1 and 7 months. Sedimentation is expressed by the ratio: height of the sediment at time x/height of the suspension at time 0.

Resuspension was obtained by turning the tube upside down, and was expressed in seconds.

pH

pH was measured with the same pH-meter previously described.

Rheology

Measurements were done with a CSL 100 rheometer (Carri-med Rheo, 91 Champlan) with a shear rate given by a geometry cone/plate (diameter 4 cm, angle 2°).

Zeta Potential

A 250 mL sample was placed in the cell of an acoustophoretic analyser Pen Kem 7000 (Noviprofibre. 38 Eybens). The zeta potential was expressed as the acoustophoretic mobility in mm/volt/sec (11).

In Vitro Diffusion

After various assays with different concentrations of NaOH added to formulas with or without glycerol, we retained the formula without glycerol. The desired conductivity was obtained by adding 1.48 g NaOH. The gel then had a pH of 7.33 and a conductivity of 9.6 mS/cm at 50 Hz. Conductivity increased from 6.4 to 11.8 mS/cm when frequencies were increased from 10 to 500 MHz. At 100 MHz, the gel conductivity was 7.1 mS/cm.

These values are comparable to those of the central part of human breast tumors. The gel rheogram has a non-Newtonian shear-thinning behaviour without yield stress. It is almost devoid of hysteresis.

500 μL or 1 mL of the suspension under study were injected perpendicularly into the gel which was left at room temperature. We observed a central area of diffusion at the injection site and a peripheral area. The length (in mm) of the greatest diameter of the central or of the peripheral area was multiplied by the length of the perpendicular diameter in order to calculate diffusion. Measurements were made at 4 and 24 h after the charcoal injection. Results were expressed in cm^2 and analysed statistically with the non-paired Student's t test. A p value <0.05 was considered significant.

In Vivo Diffusion

Charcoal diffusion was controlled macroscopically in the tumor and in the organs (kidneys, liver, spleen, lungs, heart), and later histologically after fixation.

RESULTS

Influence of the Nature of the Charcoal

Suspensions

Both charcoals were micronized to obtain an almost identical average granulometry which would allow comparisons. Table 1 indicates that 2.5–5 μm particles constituted the main

Table 1. Influence of the Nature of Charcoal on the Properties of the Suspension and the in Vitro and in Vivo Diffusion

Formulation properties granulometry	Peat		Wood
Mean diameter (μm)	6.3 ± 0.5		6.3 ± 0.3
		% of each size	
1.0–2.1 μm	21.7 ± 1 (A)		36.7 ± 1.4 (A′)
2.1–5.4 μm	62.5 ± 1.4 (B)		46.5 ± 0.6 (B′)
5.4–10.8 μm	15.7 ± 0.4		16.9 ± 0.8
Sedimentation: hu/ho % at 20 min and at 7 months	46–24		55–24
Resuspension: seconds at 48 h and at 7 months	5–7		5–6
pH	5.38 ± 0.02		6.83 ± 0.01
In vitro Diffusion			
Central Diffusion Area (cm2)			
T = 4h	1 ± 0.1		1.2 ± 0.1
T = 24h	1.1 ± 0.1		1.2 ± 0.1
Peripheral Diffusion Area (cm2)			
T = 4h	4.8 ± 0.3 (C)		8.5 ± 0.2 (C′)
T = 24h	5.8 ± 2.5 (D)		12.2 ± 1.5 (D′)
In vivo Diffusion			
Macroscopical presence Histology	in 5/5 mice in a nodule, extracellular within the tumor and in histiocytes around the tumor		in 6/6 mice in an intratumoral nodule, in spots
Intratumoral diffusion	very limited		limited

[a] $P < 0.05$ between A and A′, B and B′, C and C′, D and D′.

fraction in both preparations. Wood charcoal had a significantly higher percentage of 1–2 μm particles than peat, while peat charcoal had a higher percentage of 2–5 μm particles.

Sedimentation of the peat charcoal suspension was more rapid than that of the wood charcoal suspension but both could be equally well suspended. This is consistent with the granulometric distribution of wood charcoal particles which shifts towards smaller diameters.

The apparent viscosity of peat charcoal was higher than that of wood charcoal, possibly because the hygroscopic character of peat charcoal induces a greater hydric solvation layer.

The pH of the suspension was lower than the physiological pH and close to the tumor cell pH. Wood charcoal appears to be more negatively charged for a wider pH range (between pH 6 and 11 relative acoustopheric mobility (RAM); and as a consequence, the wood charcoal zeta potential is constant). The charge inversion point, which corresponds to a passage from a positive to a negative RAM value, is close to 2 for wood charcoal and 3.5 for peat charcoal.

In Vitro Diffusion

Central diffusion areas of peat and wood charcoal were found to be identical after 4 and 24 h but areas of peripheral diffusion were statistically greater for peat ($p < 0.005$) because of a greater amount of smaller particles (Table 1).

In Vivo Diffusion

Diffusion from the site of injection was weaker in tumors injected with the peat suspension than in those injected with the wood suspension (Fig. 1 and 2).

Based on this data, it seemed more appropriate and was thus used in the following studies. The results demonstrated a good correlation between the *in vitro* and *in vivo* diffusion.

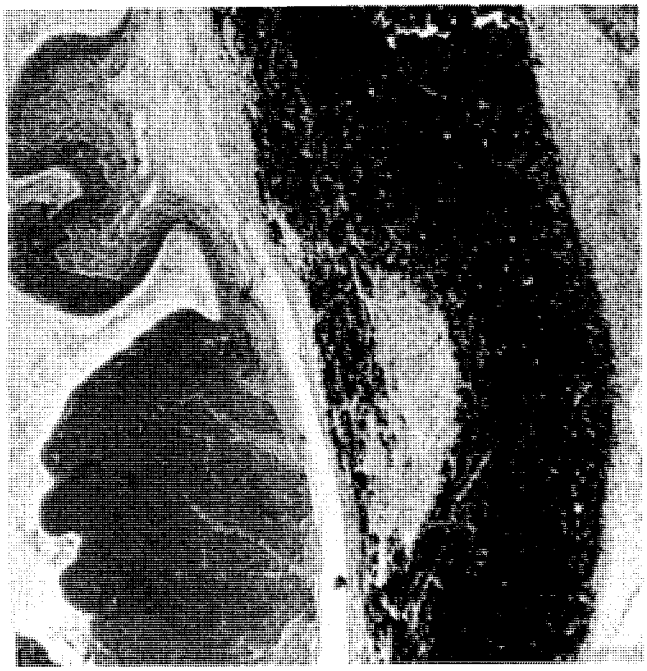

Fig. 1. Peat charcoal forms a nodule in the tumor which is not phagocyted by histiocytes. Diffusion is limited (HES × 80).

Influence of Charcoal Granulometry

Suspensions

As the size of the particles is an important factor influencing diffusion, peat charcoal was treated with different methods to obtain different granulometries.

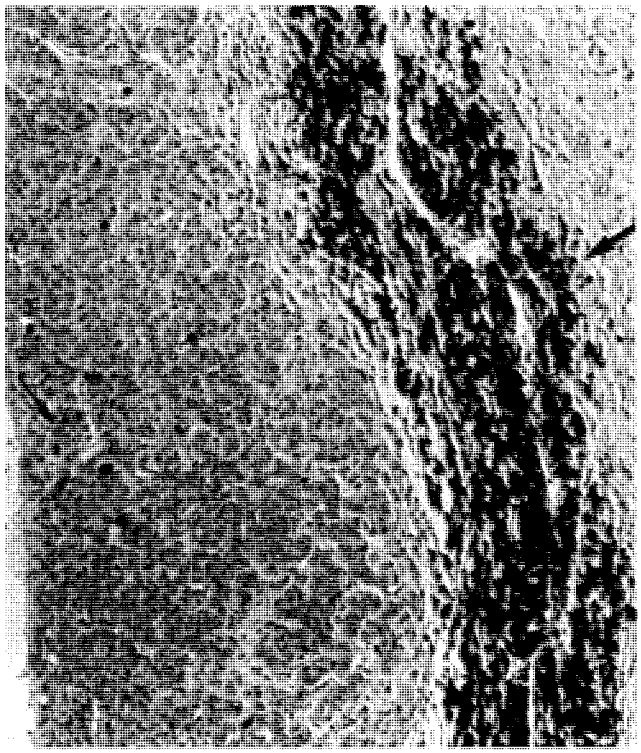

Fig. 2. Wood charcoal is localized at the periphery of the tumor (→). It is ingested by histiocytes, and mainly at the periphery (→) (HES × 80).

The following preparations were then compared: i) untreated material, ii) material obtained by micronization, and iii) material obtained by wet crushing for 20 min.

Crude charcoal contained 34% of particles measuring between 1 and 2 μm (Table 2). After micronization, only 22% of particles smaller than 2 μm were obtained. The preparation obtained after 20 min of wet crushing contained a higher percentage of small particles (71.1%). Sedimentation was faster with the untreated charcoal suspension than with the micronized one. Resuspension was obtained in a similar fashion in both cases.

The pH of the charcoal suspension prepared after dry crushing was lower (5.38), probably due to air oxidation and heating, which was higher than during wet crushing.

The surface of crude charcoal particles is positively charged when the pH is below 4, negatively charged from pH 4 to pH 9, then positively charged thereafter. Charcoal obtained by dry crushing is negatively charged for a wider pH zone.

Dry crushing reduced viscosity compared to that of crude charcoal. The structure of charcoal changes according to granulometric distribution, which itself can also be expressed as a modification in viscosity.

In Vitro Diffusion

The area of peripheral diffusion of the untreated preparation was greater than that of the micronized preparation when measured at 4 h.

The gel behaved like a dialysing system through which diffusion of the finest particles led to fractionation.

In Vivo Diffusion

Micronized charcoal remained as nodules with or around the tumor in histiocytes or outside the cells. Ingestion by histiocytes at a distance from the injection site was weak. Diffusion was less than that of crude charcoal.

Charcoal obtained by wet crushing was mainly localized around the tumor in spots and very often had been ingested by histiocytes. Considerable diffusion had occured and very little charcoal was found at histology.

Dry crushed charcoal diffused the least *in vitro* and *in vivo* (Table 2) We wondered whether the charcoal concentration could influence results.

Influence of Concentration

Suspensions

The largest average size of the particles was found in the 10% suspension which also contained the majority of the 6–12 μm particles (Table 3).

The greater the concentration, the faster the sedimentation. Resuspension was easier with the less concentrated suspension.

Variations in pH values were limited, with a slight reduction as the concentration increased. For the same apparent shear rate, apparent viscosity increased with concentration. In addition, the shear thinning character increased with concentration.

In Vitro Diffusion

We injected into the gel 0.5 mL of the 8%, 10%, and 20% suspensions and 1 mL of the 4%, 5%, and 10% suspensions. Areas of peripheral diffusion decreased when the concentration and injected volume diminished (Table 4).

After 4 hours, a significant reduction was observed in peripheral diffusion as the concentration increased and the injected volume decreased, although the amount of charcoal remained constant. The central surface area was divided by 2 when the injected volume or concentration was divided by 2, whereas peripheral diffusion was divided by 3.5 in both cases.

This could be due to a decrease in the particle diffusion coefficient with concentration, a classic phenomenon of non-Fickian diffusion for systems in which entities which diffuse may interact with each other. When 10% 1 mL and 20% 500 μL results were compared, no significant difference was found between areas of peripheral diffusion.

Interactions which limit charcoal diffusion were already saturated at the 10% concentration, which may explain why charcoal particles were transported in a similar fashion irrespective of whether the same amount was injected at concentrations of 10% or 20%.

When the injected volume was the same, the central surface area was fairly constant: 1.5 cm^2 for 1 mL and 0.8 cm^2 for 500 μL. This confirms that there is a correlation between the central surface area and spreading, and not with a mechanism of diffusion.

The area of peripheral diffusion decreased for concentrations of 4% and 10% (for injected volumes of 1 mL). In contrast, it increased for a concentration of 20%, compared to the 8% and 10% systems with the same injected volume of 500 μL.

This property should be linked to a hypothesis put forward concerning the saturation of diffusion interactions beyond 10%.

Table 2. Influence of the Mode of Grinding on the Properties of the Charcoal Suspension and the in Vitro and in Vivo Diffusion

Formulation properties granulometry	Untreated (1)	Dry crushing (2)	Wet crushing 20 min (3)
Mean diameter (μm)	7.7 ± 0.4 (A)	6.3 ± 0.5 (C)	4.8 ± 0.2 (B)
		% of each size	
1.0–2.1 μm	34.0 ± 0.8 (A)	21.7 ± 1 (C)	71.1 ± 0.3 (B)
2.1–5.4 μm	47.5 ± 1.2 (A)	62.5 ± 1.4 (C)	26.6 ± 0.1 (B)
5.4–10.8 μm	17.2 ± 0.5 (A)	15.7 ± 0.4 (C)	2.3 ± 0.0 (B)
Sedimentation: hu/ho % at 20 min and 7 months	15–12	46–24	ND
Resuspension: seconds at 48 h and at 7 months	4–6	5–7	ND
pH	6.44 ± 0.02	5.38 ± 0.03	6.35 ± 0.02
In vitro diffusion			
Central diffusion area (cm2)			
T = 4 h	1.7 ± 0.2	1.2 ± 0.1	ND
T = 24 h	2.2 ± 0.1	1.4 ± 0.1	
Peripheral diffusion area (cm2)			
T = 4 h	8.5 ± 3.0	3.5 ± 1.4	ND
T = 24 h	13.5 ± 1.1*	5.9 ± 3.3	
In vivo diffusion			
Macroscopical presence	in 4/8 mice	in 5/5 mice	in 7/8 mice
Histology	Mainly peritumoral extra and intracellular	Nodular peritumoral in histiocytes or extracellular intratumoral	extra and intracellular in macrophages, intratumoral in the necrosis ingested by histiocytes, migration away from the tumor
Intratumor diffusion	More important	Very limited	limited

Note: Statistical comparisons: *$p < 0.05$ between 1 and 2: A; 1 and 3: B; 2 and 3: C; ND: not done.

Table 3. Influence of Charcoal Concentration on the Properties of the Suspension

Formulation properties granulometry	4% (1)	5% (2)	8% (3)	10% (4)	20% (5)
Mean diameter (μm)	6.1 ± 0.3 (C)	5.4 ± 0.0 (F)	6.0 ± 0.2 (H)	7.6 ± 0.2	5.8 ± 0.1 (J)
			% of each size		
1.2–2.4 μm	21.5 ± 1.9	25.8 ± 1.6	21.4 ± 2.0	21.4 ± 3.2	23 ± 1.6
2.4–6 μm	59.2 ± 4.3	67.2 ± 1.1	64.9 ± 1.6	55.5 ± 4.2	55.5 ± 4.2
6–12 μm	19.2 ± 6.2	6.9 ± 0.6 (E) (F)	12.8 ± 1.6 (H)	23.2 ± 1.2 (J)	10.9 ± 1.1
Sedimentation: hu/ho % at 20 min and at 7 months	46–24	–	87–38	87–46	96–63
Resuspension: seconds at 48 h and at 7 months	5–7	–	9–10	10–13	–
pH	5.38 ± 0.01	–	5.21 ± 0.01	5.28 ± 0.02	
Rheology Shear rate (s-10			Apparent viscosity (Pa·s)		
10	0.031	ND	ND	0.073	0.135
100	0.010	ND	ND	0.020	0.071
1000	0.004	ND	ND	0.007	0.016

Note: Statistical comparisons: *$p < 0.05$—between 1 and 2: A; 1 and 3: B; 1 and 4: C; 1 and 5: D; 2 and 3: E; 2 and 4: F; 2 and 5: G; 3 and 4: H; 3 and 5: I; 4 and 5: J; ND: not done.

Table 4. Influence of the Charcoal Concentration and of the Injection Volume on In Vitro Diffusion (t = 4h)

Concentration and volume	Central diffusion area (cm2)	Peripheral diffusion area (cm2)
4% (1 mL)	1.5 ± 0.7	6.6 ± 1.5[a]
8% (500 μL)	0.9 ± 0.3	1.9 ± 0.4[b]
5% (1 mL)	1.3 ± 0.5	6.5 ± 1.5[a]
10% (500 μL)	0.6 ± 0.1	1.8 ± 0.4[b]
10% (1 mL)	1.9 ± 0.2[a]	3.7 ± 1.2
20% (500 μL)	1.0 ± 0.0	3.8 ± 0.5

Note: Comparison of the diffusion areas according to the concentration and to the injected volume.

[a] $p < 0.05$ Statistical comparison according to the injected volume (4% 1 mL to 8% 500 μL)(5% 1 mL to 10% 500 μL)(10% 1 mL to 20% 500 μL).

[b] $p < 0.05$ statistical comparison according to the concentration (8%–20% 500 μL) and (10%–20%–500 μL).

In Vivo Diffusion

We injected intratumorally 50 μL of the 8%, 10%, and 20% and 100 μL of the 4%, 5%, and 10% suspensions. The 4% suspension exhibited limited diffusion.

After the injection of 100 μL of the 10% charcoal suspension, a nodule was observed and some rare images of charcoal-phagocyting histiocytes were seen.

When larger volumes were injected, mechanical pressure was induced and charcoal diffusion was more intense. It also appeared that at the highest concentrations (8% and 10%), the presence of deposits of charcoal particles prevented histiocytes from ingesting them and from transporting them by migration.

A good correlation existed between in vivo gel diffusion and in vitro diffusion. From these results, the 10% concentration was selected.

DISCUSSION

From our present study, which compared the impact of the nature, the granulometry, and the concentration of charcoal preparations in vitro and in vivo diffusion, it appeared that the best formulation was micronized peat charcoal, suspended in water for parenteral injection, at a concentration of 10%. Fifty percent of particles measured between 2 and 5 μm.

The suspension we defined has satisfactory formulation. Peat charcoal was less easily phagocyted by histiocytes than wood charcoal in terms of the nature of the charcoal. This is probably because macrophages are known to ingest substrates whose surface is more hydrophobic than their own, and that wood charcoal is more positively charged than peat charcoal (13).

Granulometry studies indicated that micronized peat charcoal was the preparation which had the smallest proportions of fine particles which are preferentially phagocyted by macrophages, and thus diffused to a considerable extent. A greater amount of 1.0 to 2.1 μm particles were present in wood or in native charcoal and in the preparations obtained with wet crushing than in the preparations obtained by air jet micronization, which eliminates them.

With respect to concentration, we found that particle agglomeration at the site of injection in the form of deposits, promotes an increase in concentration which histiocytes find difficult to phagocyte.

Since the suspension is prepared to be injected intratumorally, the pH level is important. The pH of mammary adenocarcinoma of the C3H mouse, 10 to 20 days after tumor graft, varies between 6.4 to 7.1 (14).

Lower values are observed in ulcerated and necrotic areas, due to severe acidosis of the tissues, often accompanied by modifications of microvascularisation which limits diffusion. In our experience, the least diffusible suspension had the lowest pH.

The intensity of the diffusion did not seem to be correlated with the rheological properties or with the zeta potential of the suspensions. We were expecting less diffusion from the more viscous suspension, and that cellular interactions would be more limited when the charcoal particles were negatively charged. The migration of charcoal particles did not occur in vivo in the organs, and a good correlation was noted between in vitro and in vivo diffusion. We therefore selected the 10% concentration with which the injected volume can be reduced. We think that the objectives of this work: the quality, the security, and the efficacy of this formulation have been obtained.

REFERENCES

1. A. N. Papaioannou. *Europ. J. Cancer.* suppl. **1**:255–58, (1980).
2. N. Simon, G. J. Lesnick, W. N. Lerer, and L. Bachman. *Surg. Gyn. Obstet.* 134–72, (1972).
3. J. F. Egan, C. B. Sayler, and M. J. Goodman. *Cancer.* **26**:1:32–37 (1976).
4. P. Dufrane, G. Mazy, and C. Vanhaudenaerde. *JBR-BTR.* **73**:401–4 (1990).
5. D. J. Czarnecki, H. K. Feider, and G. F. Splittgerber. *AJR.* 261–9 (1989).
6. G. Svane. *Acta. Radiol. Diagn.* **24**:145–151 (1983).
7. A. J. Surowiec, S. S. Stuchly, R. Barr. and A. Swarup. *Trans. Biomed. Eng.* **35**:257–263 (1988).
8. A. P. Van Der Berg, J. L. Wike Hooley, and A. E. van Der Berg. *Eur. J. Cancer. Clin. Oncol.* **18**:457–462 (1982).
9. M. El akoum, and M. Gautherie. *Inov. Tech. Biol. Med.* **9**:91–97 (1988).
10. G. Roussy; K. Agbossou, and J. M. Thiebaut. IEEE. *Trans. Instrum and Meas.* **41**:33:66–369 (1992).
11. P. Depraetere. *Spectra 2000* **16**:132:30–36 (1988).
12. J. N. Rouzaud. *C. R. Acad. SC.* **303**, II, 12 1101–1104 (1986).
13. L. Illum, SS. Davis, RH. Müller, E. Mak, and P. West. *Life sciences* **40**:4:367–374 (1987).
14. P. W Vaupel, S. Frinak, H. I. Bicher *Canc. Res.* **41**:2008–2013 (1981).