The *in vivo* performance of biomagnetic hydroxyapatite nanoparticles in cancer hyperthermia therapy

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ABSTRACT

Hyperthermia therapy for cancer has drawn more and more attention these days. In this study, we conducted an *in vivo* cancer hyperthermia study of the new magnetic hydroxyapatite nanoparticles by a mouse model. The magnetic hydroxyapatite nanoparticles were first made by co-precipitation method with the addition of Fe^{2+}. Then, magnetic-HAP powder (mHAP) or pure HAP powder (HAP) was mixed with phosphate buffer solution (PBS), respectively. The mixture was injected around the tumor. In order to achieve hyperthermia, the mice were placed into an inductive heater with high frequency and alternating magnetic field. Only the mice which were injected with mHAP and had been treated inside the magnetic field showed dramatic reduction of tumor volume, in the 15-day observation period. No local recurrence was noted. The blood test of mice proved that mHAP powders possessed good biocompatibility and little toxicity when injected subcutaneously. Therefore, our new magnetic hydroxyapatite nanoparticles have demonstrated therapeutic effect in a mouse model with little toxicity. Further study should be done before its application inside the human body.

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1. Introduction

Magnetic nanoparticles have been used commonly for various biomedical circumstances [1–3]. Recently, the functional properties of the magnetic nanoparticles have been modified individually so that they could be used in different biological applications, such as cell label and separation [4–7], immunoassay [8,9], drug delivery [10–12], MRI contrast agents [13–18], and hyperthermia [19–21]. Iron oxide (maghemite γ-Fe_2O_3 or magnetite Fe_3O_4) is one of the most popular magnetic nanoparticles in medicine and biotechnology [22,23]. Recently, many breakthroughs of synthesis and surface engineering of iron oxide nanoparticles have been made among several magnetic particles [2]. Moreover, there are still newly-formed magnetic nanoparticles proposed (bioglass ceramics [24,25] and hydroxyapatite [26]).

Many researchers have developed different magnetic nanoparticles of ferrimagnetic bioglass ceramics (FBCs) which provide magnetic properties for MRI and hyperthermia purposes [24]. Nevertheless, during the preparation of FBC, it has been put in an oven at temperature higher than 800 °C. Then it was quenched to room temperature. This procedure would probably cause crystal growth, which made it difficult to synthesize nano-sized particles [27]. Therefore its huge size limited its own application in biomedical settings, such as injection into venous system and reaching the tumor tissue inside the animal body.

One of the mineral components inside the bone and teeth of the human body is calcium hydroxyapatite (HAP), with a chemical formula of $Ca(2)_6Ca(1)_4(PO_4)_6(OH)_2$ ($P63/m$). Therefore it possessed good biocompatibility property and adequate biodegradation rate that not only has been widely used in orthopaedics as bone grafts but also in drug delivery systems for controlled release [28–30]. The physicochemical and biological properties of apatite can be dominated by different compositions and crystal structures [31]. By particular co-precipitation with various amounts of metal ions, such as Ni(II), Co(II), Al(III), and La(III), scientists could substitute the calcium ions (Ca^{2+}) in the HAP. The magnetic nanoparticles would have different surface configurations, morphologies, and crystal architectures [32–34].

In our previous study [26], magnetic hydroxyapatite nanoparticles were fabricated by the co-precipitation process with different concentrations of Fe^{2+} added. The characteristics and biocompatibility of this newly developed magnetic nanoparticle were analysed in each sample. The particle size was around 20–50 nm with a shape of short rod or sphere. The magnetization was around 3.42–20.92 emu/g. Among all different nanoparticles proposed, the sample

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with molar ratio of Fe/Ca as 1 ($X_{Fe/Ca} = 1$) was chosen for this *in vivo* study. The ratio showed optimum crystal size with better heating rate when magnetic field applied on. It should have a better performance as a thermoseed for hyperthermia (as shown in Table 2 and Fig. 9 of reference [26]).

In this study, we try to apply the magnetic biomaterial for hyperthermia therapy in an animal model. We would like to evaluate its *in vivo* heating efficiency and tumor curing ability.

2. Materials and methods

2.1. Preparation of hydroxyapatite (HAP) and magnetic-HAP (mHAP) nanoparticles

The synthesis of hydroxyapatite (HAP) is referred to [35,36]. The reaction is as follows:

$$10Ca(OH)_2 + 6H_3PO_4 \rightarrow Ca(2)_6Ca(1)_4(PO_4)_6(OH)_2 + 18H_2O$$
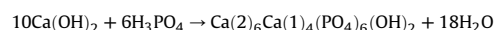
The magnetic-HAP powders were synthesized by a similar method, and it was introduced in our previous paper [26]. In brief, the steps contained addition of iron(II) chloride tetrahydrate (FeCl$_2$·4H$_2$O, Fluka, USA), adjusting pH to 8.5, stirring for 2 h followed by ageing for another 10 h, washing, and freeze-drying. The final product of a dispersible apatite powder was made. As mentioned before in the "Introduction" section, nanoparticles with molar ratio of Fe/Ca as 1 ($X_{Fe/Ca} = 1$) were chosen for the following experiments in this study.

2.2. Heating efficiency (in vivo)

In the pilot study we have tested *in vivo* heating efficiency of the material. The 0.8 g magnetic-HAP powder (mHAP) or pure HAP powder (HAP) were mixed with 5 ml phosphate buffer solution (PBS), respectively. An amount of 0.5 cc mixture was injected subcutaneously around the tumor of mice with no. 21 syringe.

In order to achieve hyperthermia, the mice were placed into a 3 cm diameter coil of the inductive heater (Power cube 64-power cube HF2; AREZZO, PRESIDENT HONOR IND CO., LTD., Taiwan). Then the heater was turned on and alternating magnetic field (60 Hz, 110 V) was generated inside the coil (47.75 G). An optic fiber was inserted inside the tumor in order to monitor temperature. The temperature was recorded by a thermometer connected to the optic fiber (Luxtron One, Lambda Photometrics, United Kingdom).

The heating efficiency was tested with different concentration of mHAP and the optimal concentration (0.8 g/5 ml) was selected. Only the body temperature of mHAP-injected mice with magnetic field raised. The mHAP-injected mice without magnetic field did not show hyperthermia effect. In the mHAP + magnetic field group, the temperature was raised to the desired temperature (45–46 °C) within 15 min. A pedal was connected to the inductive heater. Only when the pedal is pressed, electric power will be supplied to the coil. By stepping on and off the pedal intermittently, we were able to switch the magnetic field on and off. Therefore we could keep the temperature between 45 and 46 °C for 20 min without overheating the animal. In addition, we also put another optic fiber into the rectum of mice and recorded the core body temperature. During the 20 min of the hyperthermia, the core body temperature slightly raised from 38 °C to 40 °C (Fig. 1).

2.3. Animal study

A total number of 37 six-week old balb/c mice were prepared. Each mouse was inoculated with 5×10^6 murine colorectal cancer cells (CT-26 cell line). Then, we

waited for another 7–10 days and allowed tumor to grow as big as its diameter achieved 0.7–1.2 cm.

These 37 mice were divided into two categories and six groups. Mice in the first category did not receive any magnetic field; whereas those in the second category were put into the coil of the heater. The first category contained three groups: Group 1 (control group, $n = 6$), the mice were not injected with any material. Group 2 ($n = 6$), the mice were injected with HAP. Group 3 ($n = 6$), the mice were injected with mHAP.

On the other hand, the second category (with magnetic field) also contained three groups: Group 4 (control group, $n = 6$), the mice were not injected with any material. Group 5 ($n = 6$), the mice were injected with HAP. Group 6 ($n = 7$), the mice were injected with mHAP. In this category, all mice were treated with hyperthermia each day during the first three days of the experiment. Then they were put into the coil on the other day (days 5, 7, 9, 11, 13, 15).

All 36 mice were sacrificed on day 15 and blood test was performed to check the liver and kidney functions.

3. Results

During the 20 min of the hyperthermia, the core body temperature slightly raised from 38 °C to 40 °C in all six groups (Fig. 1). The core body temperature was not affected by the focal hyperthermia effect of mHAP-injected site on the back. All mice sweated during these 20 min. But they did not have symptoms of dehydration and all survived the whole experiment of two weeks.

Among the six groups, only the tumors in Group 6 (mHAP with magnetic field) shrinked significantly. The tumors in Group 1 (control group without magnetic field) grew faster than any other groups; except on days 13 and 15, the size of tumor in Group 2 (HAP without magnetic field) was larger than Group 1 (Fig. 2).

The tumors not only shrinked rapidly in Group 6 (mHAP with magnetic field), but they became black, flat and hard in days 3–5. The black area also disseminated across the back of the mouse. No
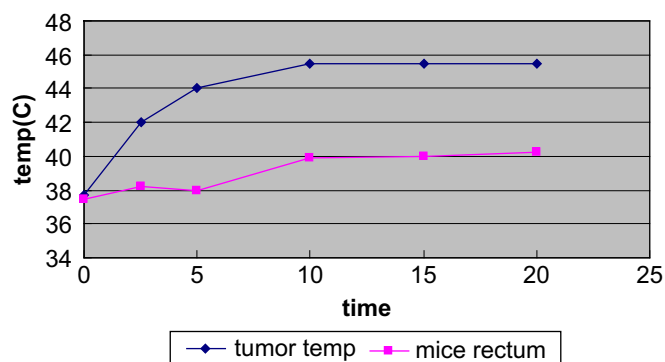
Fig. 1. The heating efficiency of mHAP. During the 20 min of the hyperthermia, the core body temperature slightly raised from 38 °C to 40 °C in all six groups.
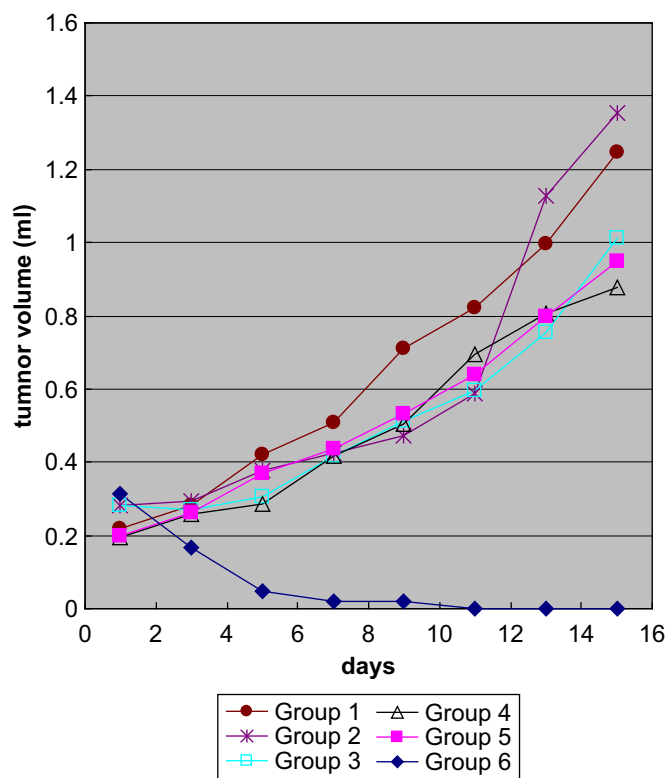
Fig. 2. The tumor size of different groups in the experimental period of 15 days. Among the six groups, only the tumors in Group 6 (mHAP with magnetic field) shrinked significantly. The tumors in Group 1 (control group without magnetic field) grew faster than any other groups; except on days 13 and 15, the size of tumor in Group 2 (HAP without magnetic field) was larger than Group 1.

skin necrosis was noted in the observation period of 15 days (Fig. 3). The tumor surface did not turn black in Group 3 (mHAP without magnetic field), suggesting that the ferrous ion deposition is not the only cause of the dark tumor surface (Fig. 4). Furthermore, in Group 4 (control group with magnetic field), central necrotic area of the tumor was noted in day 5 and enlarging till day 14. The same phenomenon was also noted in Group 1 (control group without magnetic field).
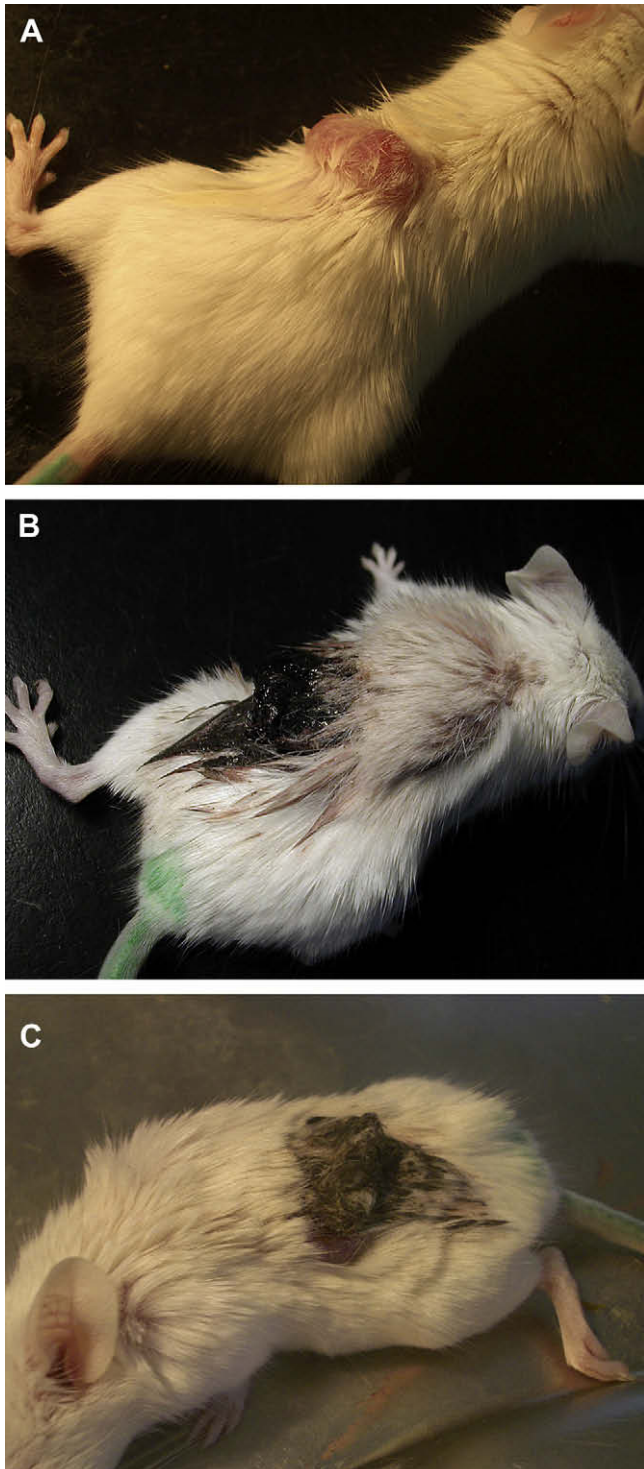
Fig. 3. The clinical photographs of the tumor in Group 6 (mHAP with magnetic field). The tumor in day 1 (A), day 5 (B), and day 14 (C).
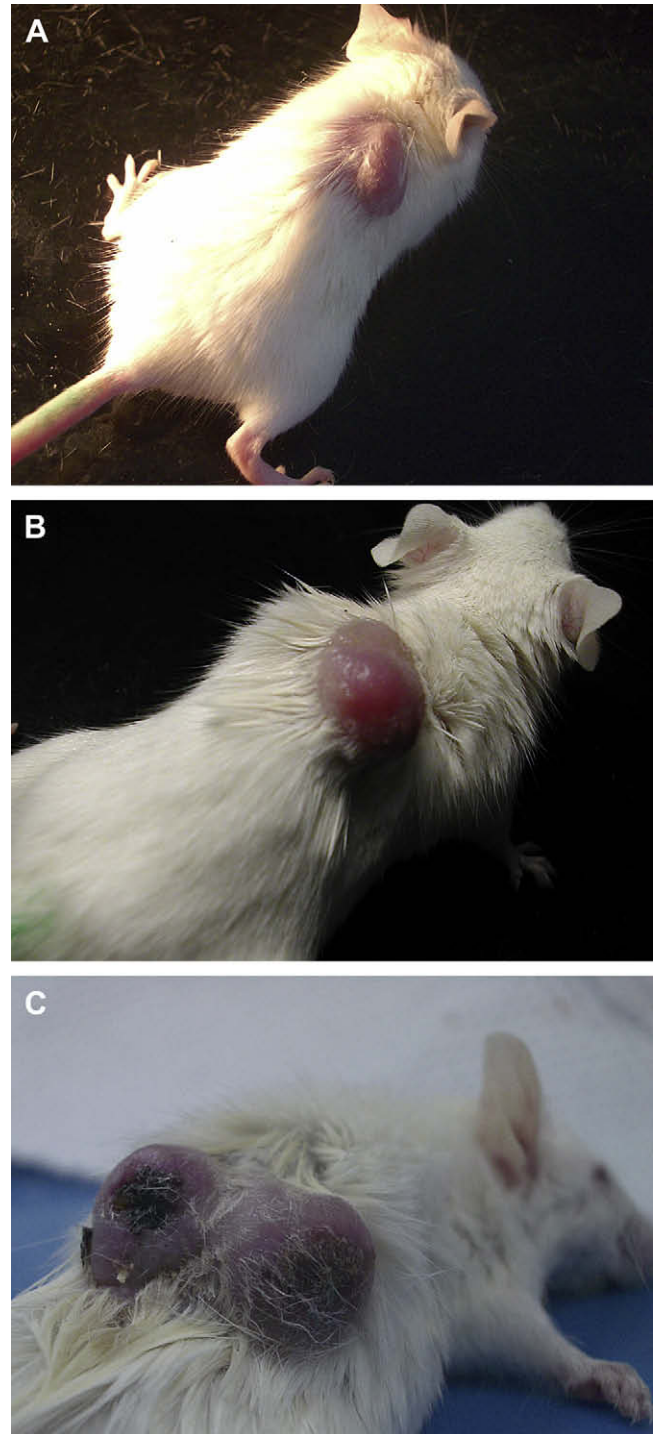
Fig. 4. The clinical photographs of the tumor in Group 3 (mHAP with magnetic field). The tumor in day 1 (A), day 5 (B), and day 14 (C).

The blood test result of liver and kidney function was listed in Table 1. All the animals in six groups have normal kidney function due to normal BUN and creatinine levels. However, all the animals have abnormal liver function due to elevated ALT and AST levels. All the animals have normal ALP level.

4. Discussion

The body temperature of the testing animals in the magnetic groups was higher than the non-magnetic groups. We thought that

Table 1
The blood test result of liver and kidney functions after the animal was sacrificed.

	BUN	Creatinine	Bilirubin	ALT[a]	AST[a]	ALP[a]
Group 1	20.67 ± 3.54	0.43 ± 0.15	10.37 ± 5.13	1626.33 ± 849.36	2289.33 ± 1755.62	20.67 ± 3.54
Group 2	28.50 ± 11.15	0.22 ± 0.08	4.18 ± 4.83	1452.00 ± 598.38	2050.80 ± 778.28	62.00 ± 38.86
Group 3	28.33 ± 16.35	0.18 ± 0.08	2.90 ± 1.31	943.50 ± 750.06	1756.83 ± 704.33	43.00 ± 28.04
Group 4	21.00 ± 5.22	0.32 ± 0.12	7.25 ± 4.82	1542.67 ± 1023.08	1694.80 ± 181.16	71.67 ± 57.03
Group 5	23.5 ± 8.78	0.25 ± 0.10	3.42 ± 3.77	710.00 ± 564.23	1390.8 ± 358.74	34.40 ± 12.97
Group 6	23.29 ± 15.82	0.34 ± 0.28	1.5 ± 1.26	2268.86 ± 1708.68	2442.67 ± 603.49	74.71 ± 43.73
Normal range	18–29	0.2–0.8	0.1–0.9	28–132	15–247	62–209

All the animals in six groups have normal kidney function due to normal BUN and creatinine levels. However, all the animals have abnormal liver function due to elevated ALT and AST levels. All the animals have normal ALP level.

[a] AST = aspartate aminotransferase (AST). AST formerly was called serum glutamic oxaloacetic transaminase (SGOT). ALT = alanine aminotransferase (ALT). ALT formerly was called serum glutamic pyruvic transaminase (SGPT). ALP = alkaline phosphatase (ALP).

it might be due to direct thermal conduction from the heated, working coil. And the ferrous material inside the mice, such as the hemoglobulin circulating in blood vessels, might also play a role in this phenomenon.

As mentioned before, the tumors shrinked and became black, flat and hard in days 3–5 (Fig. 3). The black area also disseminated across the back of the mouse. The black color was only partially contributed by repeated injection of ferrous-containing HAP and subsequent local deposition of ferrous ion, because we did not observe the same finding in Group 3 (mHAP without magnetic field) (Fig. 3). Our explanation of the disseminated black area in Group 6 is: the hyperthermia killed the tumor and created a void space. Then, the mHAP particles flowed out of the injection area and filled the new space all over the back.

In Group 4 (control group with magnetic field), central necrotic area of the tumor was noted in day 5 and enlarging till day 14. The same phenomenon was also noted in Group 1 (control group without magnetic field). Because there was no mHAP particle injected, the tumor itself should not generate heat. The central necrosis should be merely due to poor nutrition supply in a rapidly-growing tumor.

Among the six groups, only the tumors in Group 6 (mHAP with magnetic field) shrinked significantly. The tumors in Group 3 (mHAP without magnetic field) still grew rapidly, indicating that the mHAP along could not reduce the tumor volume. In other words, the shrinkage of the tumor in Group 6 was solely due to the hyperthermia effect caused by mHAP, and the mHAP along did not have the ability of killing tumor cells.

The tumor in Group 1 (control group without magnetic field) grew faster than any other groups; except on days 13 and 15, the size of tumor in Group 2 (HAP without magnetic field) was larger than Group 1. It suggested that the HAP itself might help the colorectal tumor growth after 2 weeks. However, we did not observe the same phenomenon in Group 5 (HAP with magnetic field). We thought that the elevated body temperature in the magnetic field could kill part of the tumor cells, and it neutralized the anabolic effect of HAP on tumor cells.

The blood test result of liver and kidney functions contained a lot of important information. All the animals in six groups have normal kidney function due to normal BUN and creatinine levels. However, all the animals have abnormal liver function due to elevated ALT and AST levels. It indicated that the metabolism of HAP and mHAP was mainly through liver. However, the elevated AST and ALP levels only indicated hepatitis in this point. Because all the animals survived through the whole experiment. It indicated that the liver was not seriously damaged and it was not fatal.

Moreover, the animals in the magnetic groups did not show consistent elevated liver and kidney functions, comparing with the animals in the non-magnetic groups. Therefore we could conclude that the high frequency reciprocal magnetic field we applied on the animals did not show significant impact on the liver and kidney functions.

All the animals have normal ALP levels, indicating that bone metabolism was not disturbed with the application of HAP. As stated before, the HAP was one of the important constitutions of bone and teeth. Thus we are concerned about its possible effect on the bone absorption and metabolism in the beginning.

5. Conclusion

The newly-formed magnetic-HAP powders have shown good biocompatibility and little toxicity when injected subcutaneously. Its heating efficiency under our setting was satisfactory. The core body temperature (represented by the rectal temperature) was not affected during the 20-min curing period. It showed significant and fast therapeutic effect of murine colon cancer within 2 weeks. No recurrence of tumor was noted. The future application of mHAP in the human oncology was expected. Further study is needed to be carried out in order to proof its safety and efficacy in the human body.

Acknowledgements

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