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ROS-induced HepG2 cell death from hyperthermia using magnetic hydroxyapatite nanoparticles

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Abstract

HepG2 cell death with magnetic hyperthermia (MHT) using hydroxyapatite nanoparticles (mHAPs) and alternating magnetic fields (AMF) was investigated *in vitro*. The mHAPs were synthesized as thermo-seeds by co-precipitation with the addition of Fe²⁺. The grain size of the HAPs and iron oxide magnetic were 39.1 and 19.5 nm and were calculated by the Scherrer formula. The HepG2 cells were cultured with mHAPs and exposed to an AMF for 30 min yielding maximum temperatures of 43 ± 0.5 °C. After heating, the cell viability was reduced by 50% relative to controls, lactate dehydrogenase (LDH) concentrations measured in media were three-

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Authors' contributions

CTY and FHL carried out the experimental studies. FQM and KYL generated the material assay and analysis data. CTY and JFL performed the *in vitro* data assay and analyses. CTY and FHL drafted the manuscript. CTY, Robert Ivkov, FHL and ICY revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable

fold greater than those measured in all control groups. Readouts of toxicity by live/dead staining were consistent with cell viability and LDH assay results. Measured reactive oxygen species (ROS) in cells exposed to MHT were two-fold greater than in control groups. Results of cDNA microarray and Western blotting revealed tantalizing evidence of ATM and GADD45 downregulation with possible MKK3/MKK6 and ATF-2 of p38 MAPK inhibition upon exposure to mHAPs and AMF combinations. These results suggest that the combination of mHAPs and AMF can increase intracellular concentrations of ROS to cause DNA damage, which leads to cell death that complement heat stress related biological effects.

Keywords

magnetic hyperthermia; magnetic hydroxyapatite nanoparticles; alternating magnetic field; cDNA microarray; reactive oxygen species

Introduction

Hyperthermia (HT) is one cancer treatment that applies 42 °C–46 °C for a period of time to a tumor, increasing the susceptibility of cancer cells to other agents [1–3]. A challenge encountered with HT is the difficulty to deposit sufficient and controlled energy to the tumor to reach therapeutic temperatures without damaging the surrounding normal tissues.

Magnetic hyperthermia (MHT) is among the newer techniques approved for clinical cancer treatment that offers better control of energy deposition [4]. It comprises magnetic fluids or thermo-seeds that are delivered to tumor lesions. The region is then exposed to an external alternating magnetic field (AMF) to excite the fluid or seeds producing heat for therapy [5–8].

Hepatocellular carcinoma (HCC) remains an ongoing global challenge to clinical management [9, 10]. HCC treatments include surgical resection, radiofrequency ablation, and liver transplantation. Clinical management of HCC is often problematic because patients present with advanced disease at the time of diagnosis. Thus, multi-modal therapies, such as chemo-HT or radio-HT, are utilized to improve treatment outcomes [11].

In prior work, we have developed several types of Fe-precipitated ceramic nanoparticles as thermal seeds for MHT [12, 13]. Among these, we have demonstrated that magnetic hydroxyapatite nanoparticles (mHAPs) provide a suitable platform for delivering cancer therapeutic agents because of their favorable biocompatibility. There are, however few reports describing how MHT induces cancer cell death [14–17].

MHT cancer treatment applied uniform temperature of the tumor tissue to above 43 °C and decreased tumor size. Amount of heat shock proteins (HSPs) is expressed within and around the tumor tissue to protect protein degradation have been reported. The levels of certain HSPs cause cancer cells thermal resistance and cell survival after HT treatment. Those genes expressions associated with cancer cells survived after MHT [18, 19]. However, there are no definitive reports were available on how MHT induces cancer cell death [14–17]

In this study, we explored the utility of mHAPs to generate cytotoxic heating upon AMF exposure in HepG2 cells. We used cDNA microarrays for whole genome screening and IPA software (Ingenuity Pathway Analysis) to investigate the mechanism of cancer cell death following MHT. Our findings suggest that ATM and GADD45 signaling might be the canonical pathways involved in the response to mHAP-induced MHT exposure of HepG2 cells.

Materials and methods

Preparation of hydroxyapatite (HAPs) and magnetic-HAPs (mHAPs)

Uniform-sized mHAPs were synthesized by co-precipitation using methods previously reported, and briefly described [20]. 0.3 M H_3PO_4 was added to a 0.5 M $\text{Ca}(\text{OH})_2$ solution until pH 8.5 was achieved, then aged for 20 h. The mHAPs produced for following steps addition of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, adjusting pH 8.5, stirring for 2 h followed by aging for 10 h, PBS washed twice. The synthesized mHAPs were characterized by x-ray powder diffractometry (XRD, Rigaku, Pint-2000, Japan), transmission electron microscopy (TEM, Philips/FEI Tecnai T20, USA) and scanning transmission electron microscopy (STEM, Philips/FEI Tecnai F20, USA) for crystal structure identification, morphology examination, and particle size analysis, respectively. The mHAPs were evaluated under $\text{Cu K}\alpha$ ($\lambda = 1.5406 \text{ \AA}$) with 40 mA and 40 kV within the 2θ range of 20° – 60° , at a rate of $10^\circ \text{ min}^{-1}$. The Scherrer's formula was used to calculate the average crystallite size of the samples. The crystalline sizes analysis was presented by benchtop x-ray diffraction (XRD) instrument (XRD, Rigaku, benchtop XRD instrument, Japan).

$$D = \frac{K\lambda}{\beta \cos \theta},$$

where: d = coherent diffraction domain size, λ = the wavelength of the x-ray source applied (1.540 59), β = the reflection width (2θ), θ = the Bragg angle and K = the shape constant (0.9) [21].

Zeta potential analysis

Size and zeta potential of the synthesized nanoparticles were obtained with a Zeta-sizer (Malvern Zetasizer Nano ZS90, Worcestershire, UK). The analyzed nanoparticles were suspended in ddH₂O, transferred to a quartz cubic cell, and zeta potential was measured. The measurement was carried out using a HeNe laser, 633 nm as light scattering ($\theta = 173^\circ$) in water at 25 °C and the measured sizes were reported using a percent volume distribution.

Evaluation of magnetic property

The synthesized mHAPs were loaded in a sample straw for super-conducting quantum interference device (SQUID) analyzed at 300 K in DC mode (Quantum Design, physical Property Measurement System, USA). The hysteresis loop was measured by applying a magnetic field varying from 3×10^{-9} to 3×10^9 Oe.

Heat generation

Heat produced by the mHAPs nanoparticles was measured using an AMF. Briefly, 0.25, 0.5 and 1 mg of mHAP nanoparticles were suspended in 1 ml of ddH₂O in a 1.5 ml centrifuge tube. The tube was placed into the center of copper coil and exposed to an AMF generated in the coil by passing an AC current from a power supply (Power cube 64/900, 750–1150 kHz, Ceia Company, Arezzo, Italy), beginning at room temperature. After the power supply was turned on, sample temperatures were measured with an optical fiber probe (I652, Luxtron Corp, USA) and recorded every 30 s for 10 min.

MHT of mHAPs to HepG2 cells in vitro

For the *in vitro* MHT study, HepG2 cells were harvested from the dishes to create a 1×10^6 cell/200 μ l suspension in MEM-alpha medium and 0.2 mg mHAPs were added in Eppendorf tube. The Eppendorf tube was placed into the center of an AMF coil (Power cube 64/900, 750–1150 kHz, Ceia Company, Arezzo, Italy), and the power was turned on. The magnetic field had a frequency of 750 kHz and 10 Oe field strength (H). The mHAPs were co-cultured with HepG2 cells and produced therapeutic temperature by AMF. The laboratory environment uses room temperature to maintain stability of the MHT experiment. During the MHT, the sample temperature remained at 43 ± 0.5 °C and the power supply was switched on/off with a foot pedal. Temperatures were measured with optical fiber probe (I652, Luxtron Corp, USA) elevating to 43 ± 0.5 °C for 30 min to perform MHT [22].

Evaluation of cell viability (WST-1 assay) and cytotoxicity (LDH assay)

Cell viability and toxicity assays of HepG2 cells were evaluated by a PreMix WST-1 (Promega, Madison, USA) assay and LDH detection kits (Promega, Madison, USA), respectively. For examining cell viability, HepG2 cells were seeded onto a 96-well culture dish with a density of 3×10^3 cells/well. Then 100 μ l of culture medium was added to each well, followed by the addition of mHAPs at a ratio of 0.1 mg/well. After 24 h incubation, 10 μ l of WST-1 reagent was added to each well at 37 °C and left for 2 h. Cell viability was evaluated by an ELISA reader (TECAN, Sunrise, Switzerland) at 540 nm. For measurement of cytotoxicity, 3×10^3 HepG2 cells were seeded onto 96-well petri dish with addition of 100 μ l of culture medium and 0.1 mg of mHAPs to each well. After 24 h, 50 μ l of culture medium from each well was collected and transferred to another 96-well culture plate. 50 μ l of LDH reagent was then added to each well and the solution incubate at room temperature for 30 min. After the reaction was complete, absorbance at 490 nm was measured by an ELISA reader. The experiment was divided into four groups (table 1). Control group means HepG2 cells only; HepG2-mHAPs means HepG2 cells only cultured with mHAP nanoparticles; HepG2-AMF means HepG2 cells treated with AMF only; HepG2-MHT means HepG2 cells treated with mHAPs nanoparticles and AMF, such as a combination treatment.

Live/dead cells staining and quantification

1×10^5 of MHT-treated HepG2 cells were collected into a 15 ml tube with 100 μ l of live/dead cells staining. The tube was wrapped in aluminum foil to block incoming light and kept at room temperature for 30 min. The stained HepG2 cells were then mounted on a cover

slide and examined under a confocal microscope (Olympus, FluoView FV300, Japan). Live cells were green, whereas dead cells were red. Quantification of the Live/Dead cells staining was performed using Image J.

Annexin V/PI staining

1×10^5 cells/ $100 \mu\text{l}$ of MHT-treated HepG2 cells were transferred to a 5 ml culture tube, and $5 \mu\text{l}$ of Annexin V and $5 \mu\text{l}$ of PI dye (FITC Annexin V Apoptosis Detection Kit I, BD Pharmingen, USA) were added. The tube was gently vortexed and kept in the dark for 15 min. Then $400 \mu\text{l}$ of 1X binding buffer was added to complete the reactions, and the tube was finally analyzed by flow cytometry (Cytomics FC500, Beckman Coulter, Indianapolis, USA). The full gating strategy is presented for flow cytometry analysis.

Reactive oxygen species (ROS) assay

The MHT-treated HepG2 cells were seeded onto a 96-well black plate with a density of 1×10^5 cells/well, then $200 \mu\text{l}$ /well of 2,7-dichlorofluorescein diacetate (DCFH-DA) was added and the tube kept in an incubator for 4 h. The intracellular ROS of MHT-treated HepG2 cells were measured by ELISA reader at 535 with 485 nm excitation.

cDNA microarray analysis and signal pathway analysis

RNA was extracted from HepG2 cells and MHT-treated HepG2 cells by RNA extraction kit (Qiagen, Hilden, Germany). RNA of $0.2 \mu\text{g}$ from each sample was amplified using the 'low Input Quick-Amp Labeling kit' (Agilent Technologies, California, USA) and labeled with Cy3 during the transcription process. Fragmented labeled cRNA was correspondingly pooled and hybridized to Agilent SurePrint G3 Human V2 GE 8×60 K Microarray (Agilent Technologies, California, USA) at 65°C for 17 h. Analysis microarray data using IPA software (Qiagen, Hilden, Germany) performed to characterize the gene expression profile after HepG2 cell treated with MHT. The significance of the functional and the signaling pathways was determined using Fisher's exact test.

Quantitative real time PCR (qPCR)

Total RNA was extracted using the TRIzol reagent (Qiagen, Hilden, Germany) and $2 \mu\text{g}$ of RNA was reverse transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). qPCR was performed in a FastStart universal SYBR Green Master (Roche, Basel, Switzerland) on individual genes. The gene-specific primer sequences showed in table 2 and GAPDH as an internal control.

Analysis of Western blot to detect apoptosis-related proteins

MHT-treated HepG2 cells were lysed with RIPA buffer. The lysates were collected in a centrifuge tube and denatured in water at 95°C . The denatured lysates of 20 mg were loaded onto gels (12% SDS–polyacrylamide) and separated by electrophoresis. Blots were probed and incubated at 4°C overnight with rabbit monoclonal antibody, detail as table 3.

Statistical method

One-way ANOVA was used to determine the statistically difference among the treatment groups, with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

mHAPs and heating by AMF

The morphology of the synthesized mHAPs was examined by TEM. We observed that the mHAPs comprised a mixture of hydroxyapatite and iron oxide magnetic nanocrystallites with aggregation form. The HAPs were a plate-like structure and magnetite were a round shape as shown in figure 1(a). High-resolution TEM images revealed crystal diffraction patterns at orientations of (220) with d -spacing of 0.29 nm adhered onto larger crystals at orientation of (211) with d -spacing of 0.28 nm, suggesting magnetite nanocrystallites adhered onto hydroxyapatite crystallites (see figure 1(b)). Compositionally, the two major crystals that appeared in the synthesized nanoparticles were hydroxyapatite and the magnetite. The major peaks of hydroxyapatite at the 2θ of 25.8° , 31.7° and 39.6° corresponded to the crystal forms of (002), (211) and (130), respectively. The characteristic peaks of 2θ at 35.5° , 43.1° and 57.0° were assigned as crystal forms of (311), (400) and (511) of magnetite, respectively. XRD pattern showed the mHAPs underlined with standard patterns of hydroxyapatite and magnetite (JCPDS cards of 82–1943 and 75–0033), see figure 1(c). The lattice plane of HAPs and iron oxide magnetic show strong diffraction peaks in (211) and (311), and presented grain sizes of the HAP and iron oxide magnetic were 39.1 and 19.5 nm which were calculated using the Scherrer formula. The electronic diffraction pattern of the mHAPs (see inset figure 1(c)) revealed a combined ring pattern composed of phases of hydroxyapatite and magnetite. Each ring was assigned to one crystal family corresponding to a peak from JCPDS cards, and the results were matched to XRD patterns as figure 1(c).

Concentration dependent heating curves of the mHAPs suspended in 1 ml MEM- α with AMF is shown in figure 1(d). 1 mg of mHAPs in 1 ml ddH₂O could elevate temperatures to 42°C within 4.5 min using the applied AMF conditions, suggesting a potential utility for cancer HT. The mHAPs concentration is 1 mg ml^{-1} . The heat generation efficiency, specific loss power (SLP) of mHAPs was defined by the following equation [23–25]:

$$\text{SLP} = \frac{C}{m_{np}} \frac{\Delta T}{\Delta t},$$

where C means specific heat capacity of water ($4.18\text{ J g}^{-1}\text{ K}$), m_{np} is the mass of the mHAPs and dT/dt is the initial slope of temperature versus time. The heating curves showed that the mHAPs under AMF increased 26.1°C within 10 min. The SLP value of mHAp was 181.8 W g^{-1} .

The magnetic properties of mHAPs was analyzed by a SQUID, as shown as figure 2. The M – H curve was obtained from an AMF of 9 T strength and temperature of 300 K. The

saturation magnetization of mHAPs is 28.6 emu g^{-1} and shows superparamagnetic properties.

The high sensitive EDS accessory to STEM was used to map the spatial distribution of P, Ca, and Fe in the mHAPs, as illustrated. The three crystals were individually precipitated without overlapping spatial distribution. EDX show Fe, Ca and P (47%, 32% and 27%) were major components in mHAPs as STEM results. (figures 3(a), (b)). The mean hydrodynamic diameter of the composite mHAPs, measured by light scattering was $689 \pm 155 \text{ nm}$ with 0.46 PDI, indicating a narrow size distribution range. The measured zeta potential of the nanoparticles was $-38 \pm 5 \text{ mV}$ as shown in figures 3(c), (d).

The in vitro study of MHT

When HepG2 cells were incubated with the mHAPs, no appreciable effects on cell viability and membrane integrity, measured by LDH assay were observed. When cells incubated with the mHAPs were exposed to an AMF, we measured a significant decrease in viability and increased LDH release in media 24 and 72 h after exposure (see figures 4(a), (b)). The difference of cell viability (WST-1) among HepG2 only group, HepG2-mHAPs group and HepG2-AMF group was less than 15%. However, there is a 50% decrease in cell viability of HepG2-MHT group by WST-1 assay as figure 3(a). The cell viability (LDH) of HepG2 cells was significantly inhibited after MHT and the cytotoxicity results were similar to those of cell viability as figure 4(b). The results were further confirmed by live/dead cells staining as shown in figure 4(c), where live cells show as green and dead cells as red with confocal microscopy. Quantification of the imaging data showed a 35% area of the field of view was red in samples recovered following MHT, compared to 5% for the controls.

Annexin V/PI staining and ROS generation

The number of HepG2 cells undergoing apoptosis following MHT was evaluated by flow cytometry with Annexin V and PI staining, respectively (see figure 5(a)). No significant differences in the ratio Annexin V positive over PI-positive populations were observed among all control groups, but MHT-treated cells (HepG2-MHT group) displayed a significant decrease of the Annexin V positive cells with corresponding increase of PI-positive cells.

Exposure to heat shock or stress can induce production of ROS in mammalian cells that can eventually lead to cell death [26]. In this study, ROS generation level of control groups (HepG2 cells only, HepG2 cells with mHAPs only and HepG2 with AMF only) shows no difference. However, HepG2 cells treated with MHT (mHAPs plus AMF) shows ROS level rise up, especially HepG2 with MHT 30 min group. 30 min following MHT with mHAPs, we detected evidence of significantly elevated ROS than in controls. These results indicate that ROS generation occurs in MHT-treated HepG2 cells with dose dependent.

Identification of gene expression and related pathway

Our observations of significant cell damage induced by the combination of mHAP and AMFs prompted us to further evaluate cell responses using gene expression analysis on HepG2 cells treated with MHT. Differences in mRNA expression between HepG2 control

and HepG2-MHT groups were evident in the Agilent human cDNA microarray shown in figure 5. In microarray assay, 29 279 genes were identified as activated, and among those, 2709 genes were found to have more than a 2-fold change (see figure 6). It was further identified that 1766 genes were up-regulated and 943 genes were down-regulated. Using the IPA software package we investigated the differentially expressed genes in the MHT-treated HepG2 cells. The top 10 canonical pathways identified from the IPA analysis are indicated in table 4.

We discovered from our analysis that the two most likely pathways that included both DNA damage and cell death were ATM and GADD45 signaling. The cell cycle regulatory gene (CDKN1A) and DNA damage genes, GADD45a, GADD45b, and TP53, also overlap with ATM and GADD45 signaling. From our IPA analysis results we determined that DNA-damage related genes, including ATM, TP53, CDKN1A, GADD45a, GADD45b, and GADD45r, were down-regulated. We further validated independently the mRNA expression of ATM and GADD45a in MHT-HepG2 group and compared this to the HepG2-only control by qPCR. As shown in table 5, both ATM and GADD45a were down-regulated (−3.058 and −4.329, respectively).

p38 kinase response to HepG2 cells treated with MHT

Our IPA results indicated that the p38 mitogen-activated protein kinase (MAPK) major linked the genes we identified from the microarray data. p38 MAPK plays a key role in the cascade of ATM signaling and GADD45 signaling [27, 28]. Hence we analyzed proteins in the p38 pathway by Western blot analysis. As shown in figure 7, the p38 MAPK cascade proteins were found to be significantly decreased after MHT. Mkk3/Mkk6, an upstream protein in the pathway, and the downstream transcription factor, ATF-2 (Activating transcription factor) were both down-regulated. Furthermore, observed that the downstream p38 effector protein—HSP27 (heat shock protein 27) was inhibited by MHT.

Discussion

For this study, we synthesized mHAPs by co-precipitation and we evaluated their biocompatibility and heating capability *in vitro* using HepG2 cells. Previous studies had demonstrated that mHAPs show similar results with lung cancer cells, A549 [13]. Our interpretation of the XRD pattern suggests that the mHAPs were successfully combined to a ring pattern of hydroxyapatite and magnetite. The preferred orientations of the crystal forms of hydroxyapatite and magnetite were (211) and (311) with were 39.1 nm and 19.5 nm were calculated by the Scherrer formula, respectively. The TEM images of the morphology of synthesized mHAPs demonstrated that iron oxide magnetic crystallites overlapped with HAP nanocrystallites.

To effect heating of the mHAPs, we exposed them to an AMF to characterize the heat generation, and effects of mHAP-induced hyperthermia (MHT) on HepG2 cells. Exposure of cells to heating with AMF+mHAP combinations was associated with evidence of increased cell stress and death, and reduced cell viability as measured by ROS and LDH levels, by live/dead staining, and WST-1 assays, respectively. Flow cytometry analysis with

Annexin V/PI staining also showed significant differences between mHAP + AMF (MHT) samples and controls.

ROS has been reported to be related with several cellular events including DNA damage and apoptosis [26, 29]. In this study, the high level ROS was induced by MHT with time depend. The HepG2 cells endure heat stress from MHT with time dependent and HepG2 cell death increased as the ROS level increases.

To determine other pathways potentially involved, we analyzed the HepG2-MHT-treated cells by cDNA microarray for gene regulation. This analysis subsequently showed that DNA-damage related genes such as ATM/ATR and GADD45a were down-regulated after MHT.

ATM and ATR pathways are both involved in the DNA damage response, and include downstream activation/stabilization of p53, p21 and GADD45 (GADD45a, GADD45b and GADD45r) families [30, 31]. ATM/ATR pathways have been observed to be significantly involved in DNA damage sensing, activating p53 and CHK-2 genes when exposed to ROS stress. It has been shown that p53-mutated cancer cells exhibit a higher sensitivity to ROS stress and ATR/ATM inhibitors [32]. However, tumor cells having low stress levels can still survive ATR/ATM inhibition. Thus, ROS level and multiple gene mutations interact and play an important role to determine the fate of cancer cells without a single factor being the sole determinant of cell death [13]. In this study, we demonstrated that HepG2 cells displayed high intracellular ROS levels and down-regulated ATM and GADD45a genes following MHT with mHAPs. These results suggest that MHT can induce a strong thermal stress that initiates downregulation of ROS-response genes, leading to HepG2 cell death.

We have demonstrated that levels of p38, Mkk3, Mkk6 and AFT-2 were also down-regulated. p38 MAPK plays a role as a regulator of cell death, in a specific manner that depends on both the type of stress and type of cell. Mkk3 and Mkk6 are important p38 upstream factors. Physical and chemical stressors (e.g., ROS, hypoxia and UV) can activate MAPKKs initiating Mkk3 and Mkk6 phosphorylation. p-38 also activates the downstream protein—AFT-2 to regulate cell survival.

Conversely, GADD45 proteins are involved in cell cycle arrest during DNA repair, cell survival and apoptosis; and, these are believed to interact with members of the MAPKs [27, 33]. Furthermore, the major components of MAPKs were ERK1/2 (p44/p42) [34], c-Jun N-terminal kinase (JNK) and p38, which are involved in various extracellular stimuli to control a large number of fundamental cellular processes including proliferation, differentiation, survival, and apoptosis [4, 28, 34]. In the Western blot assay, we observed that p38 MAPK was strongly suppressed by MHT. The p38 upstream kinase Mkk3/Mkk6 [35] and downstream transcription factor ATF-2 were inhibited by MHT. The inhibiting effect of MHT, like the p38 inhibitor SB202190 [36, 37], was even propagated to proteins such as HSP27. We previously used the mHAPs as a thermos-seeds with AMF to perform an in vivo study with local MHT. The results showed that it significantly decreased the tumor volume [12]. However, the mechanism of how MHT kills cancer cells has not been investigated. Our

results suggest a possible mechanism of mHAPs-MHT-induced death of HepG2 cells may occur via DNA-damage repair genes and MAPK signaling.

The colon tumor and lung tumor regression in xenografts in a nude mice model had been presented on the combination cancer treatment of mHAPs and AMF [12, 13]. mHAPs were presented intratumoral injection plus AMF and tumor regression is observed. However, the death mechanism for cancer cells is unclear. In this study, the HepG2 cell death was performed and expected to provide a high efficiency MHT cancer treatment. Further work is needed to explore the nature of these effects in other cells and animal models to determine the potential clinical benefit.

Conclusion

The combination of exposing HepG2 cells to mHAPs and AMFs to induce MHT was associated with increased ROS levels and activation of DNA-damage repair genes (ATM and GADD45a). Generally, HT and MHT can act through multiple pathways to kill cancer cells. We present tantalizing evidence that HepG2 cells exposed to mHAP-induced MHT produces elevated intracellular ROS levels, and subsequent DNA damage. This study provides motivation for further exploration of using mHPAs with AMFs as a means to treat HCC with MHT. rationale for developing MHT for clinical treatment of HCC.

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Figure 1.

Synthesized magnetic-HAPs and generated heat by AMF. (a) The TEM image of synthesized mHAPs. HAPs were plate-like structures (blue arrow) and magnetite is a round shape (red arrow) was precipitated onto the surface of HAPs. (b) From the images of high-resolution TEM (HRTEM) as figure 2(b), the magnetite (220) with d-spacing of 0.29 nm was precipitated onto hydroxyapatite (211) with d-spacing of 0.28 nm. (c) XRD pattern of the mHAPs was in agreement with the standard patterns from JCPD cards of 82–1943 and 75–0033; those were hydroxyapatite and magnetite, respectively. The upper right corner showed the electronic diffraction pattern of synthesized mHAPs. (d). The heating curve of different amount of mHAP in 1 ml ddH₂O induced under AMF. Based on the heating profile, 1 mg group should have a better heating efficiency than that of 0.25 mg group and 0.5 mg group. The experiment showed that 1 mg of mHAP in 1 ml ddH₂O could elevate temperature up to 42 °C within 4.5 min under AMF induction; that could provide enough thermal stress for cancer HT.

Figure 2. mHAPs show superparamagnetic properties. The curve of magnetization and applied magnetic field (M - H curve) for mHAP nanoparticle, indicated in red circles.

Figure 3.

Synthesized magnetic-HAPs contain Iron oxide having inverse spinel structure. (a) The high sensitive EDS accessory to STEM to map the spatial distribution of P, Ca, and Fe in the mHAP nanoparticles as illustrated. The three crystals were individually precipitated without overlapping spatial distribution. (b). EDX show Fe, Ca and P (47%, 32% and 27%) were major components in mHAPs as STEM results. (c) The average particle size of the synthesized mHAP is 689 ± 155 nm with 0.46 of distribution index PDI that tells the nanoparticles in a narrow distribution range in adequate homogeneity. (d) The surface potential of the nanoparticles was -38 ± 5 mV that was above stable surface charge and could be stable storage in aqueous solution without serious aggregations.

a. WST-1 assay

b. LDH assay

c.

Figure 4.

The *in vitro* study of magnetic hyperthermia (MHT). (a) WST-1 presented HepG2 cells treated with different conditions on 24 and 72 h. HepG2 cells show no regrow after MHT. Conversely, HepG2 cells treated with mHAPs only and AMF only, the cancer cells grow rate same as control group. (b) A lot of dead cells in HepG2 with MHT group was presented in LDH assay. (c) Live and dead cells of HepG2 were in green and red in live/dead cells staining. The red spots (35%) were observed after HepG2 cells treated with MHT.

Figure 5.

Annexin V/PI staining reactive and reactive oxygen species (ROS) generation. (a) The percentage of Annexin V positive population and dead cells in each experimental groups. The results of Annexin V/PI staining of HepG2 cells treated in different conditions were checked by flow cytometry to show the population of apoptosis and dead cells. In HepG2 with MHT group, Annexin V positive cells were decrease and dead cells were increased noticeably. (b) The ROS level of HepG2 cells were detected by different treatments after 4 h. ROS levels of HepG2 with MHT 30 min was higher than MHT 10 min. The more ROS was generated by longer MHT treatments. The ROS level of HepG2 cells with mHAPs only and AMF only (10 min or 30 min) were no different with control group.

Figure 6.

The control group gene expression had a large shift to HepG2 cells with MHT treatment. Logarithmic scatter-plots comparing expression profile for control and HepG2-MHT groups. The genes expression analyzed by microarray and fluorescence signal strength values in X axes and Y axes represented HepG2 only group and HepG2-MHT. Each probes presented of a gene chip hybridization signal. The plots of gene had dramatically shifted to X axes. The genes level in HepG2 with MHT treatment got a significant change.

Figure 7.

p38 kinase response to HepG2 cells treated with MHT p-P38 protein level was found to be significantly decreased after MHT treatment. The p38 cascade are of Mkk3/Mkk6, ATF-2 show dramatically inhibited in process in phosphorylation. P-HSP27 protein level show strongly inhibited by MHT treatment. Those proteins related to p38 pathway were downregulated after HepG2 cells with MHT; the β -actin was as the internal control in Western blot.

Table 1.

List of experimental groups.

Experimental group	Details
HepG2 only	HepG2 cells without any treatment
HepG2-mHAPs	HepG2 cells treated mHAPs
HepG2-AMF	HepG2 cells treated with only alternative magnetic field without mHAPs
HepG2-MHT	HepG2 cells treated with mHAPs and applied with AMF

Table 2.

List of experimental groups.

ATM	F: 5'-CTGGAGGAAGTGTCTCAGCAAAAG-3'	R: 5'-AGAGCCACATCTCTGTCTCGTGT-3'
GADD45-alpha	F: 5'-CTGGAGGAAGTGTCTCAGCAAAAG-3'	R: 5'-AGAGCCACATCTCTGTCTCGTGT-3'
GAPDH	F: 5'-GGCAGCAGCAAGCAATTCCT-3'	R: 5'-GCCCAACACCCCCAGTCA-3'

Table 3.
The Western blotting of phospho-specific antibodies show as table 3. The β -action used as an internal control.

Antibody	Phosphorylation sites	Dilution	Sources
p-p38 MAPK	Thr180/Tyr182	1:1000–1:1500	Cell signaling
p-p44/42	Thr202/Tyr204	1:1000–1:1500	Cell signaling
p-Mkk3/Mkk6	Ser189/Ser207	1:1000–1:1500	Cell signaling
p-ATF-2	Thr71	1:1000–1:1500	Cell signaling
P-HSP27	Ser82	1:1000–1:1500	Cell signaling
β -actin		1:1000	Cell signaling

Table 4.

Top 10 IPA canonical pathways for MHT treatment and expressed genes in HepG2 cancer cells.

Ingenuity canonical pathways	−log (p-value)	Molecules
IL-17 signaling in gastric cells	3.88E+00	CCL5, CXCL1, CXCL8, EGFR, JUN, TNF
Granulocyte adhesion and diapedesis	3.77E+00	CCL5, CXCL1, CXCL2, CXCL3, CXCL8, CXCL12, HRH1, IL1RAP, MMP7, SDC2, TNF
ATM signaling	3.52E+00	CDKN1A, GADD45a, GADD45b, JUN, NFKB1A, TP53
Agranulocyte adhesion and diapedesis	3.11E+00	CCL5, CXCL1, CXCL2, CXCL8, CXCL12
Molecular mechanism of cancer	3.03E+00	AKT2, BAX, BCL2L1, CDKN1A, FOXO1, IRS1, JUN, MYC, NFKB1A, SMAD2, SMAD7, SMAD9, TGFB2, TP53, WNT10B, WNT2B
GADD45 signaling	2.67E+00	CDKN1A, GADD45a, GADD45b, TP53
Colorectal cancer metastasis signaling	2.56E+00	AKT2, BAX, EGFR, JUN, MMP7, MYC, SMAD2, STAT3, GFB2, TNF, TP53, WNT10B, WNT2B
Telomerase signaling	2.54E+00	AKT2, CDKN1A, EGFR, MYC, SP1, TP53
T helper cell differentiation	2.54E+00	IL5, IL6, IL13, STAT1, STAT4, IL12R, IFN γ
Ephrin receptor signaling	2.52E+00	AKT2, CXCL12, LIMK1, SDC2, STAT3

Table 5.

List of candidate genes up-and down-regulated mRNAs.

Systematic name	DNA damage signal	Gene symbol	Gene name	Fold change	
				Array	qPCR
NM_000051		ATM	Ataxia telangiectasia mutated	-2.238	-3.06
NM_000546		TP53	Tumor protein p53	-2.08	
NM_078467		CDKN1A	Cyclin-dependent kinase inhibitor 1A	-2.18	
NM_001924		GADD45a	Growth arrest and DNA-damage-inducible, alpha	-2.14	-4.33
NM_015675		GADD45b	Growth arrest and DNA-damage-inducible, beta	-2.42	
NM_006705		GADD45g	Growth arrest and DNA-damage-inducible, gamma	3.00	