MINI REVIEW

Received: September 2018
Accepted: December 2018

A Review on the Application of In Vitro and In Vivo Models of Cancerous Tumors for the Study of the Hyperthermia Effect

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BCCR
2019; 11(1):71-81
www.bccrjournal.com

ABSTRACT

Hyperthermia is a novel method for cancer therapy. To have the best control when heating tissues in hyperthermia, the use of magnetic nanoparticles is suggested. The local control of heat is very important in this technique, to prevent the damage of healthy tissues around the tumor, and therefore it is necessary to measure changes in temperature to determine the optimum conditions in which hyperthermia can create the desired results. The type and concentration of nanoparticles and nanoparticle distribution within the cancerous tissue are key factors affecting temperature distribution throughout the hyperthermia process. One of the main factors influencing nanoparticle distribution is the characteristics of the diffusion media, such as chemical composition, morphological and mechanical features, all of which affect the diffusion of nanoparticles at the cancer site. In this review, the most common in vitro and in vivo media and their influence on the results of hyperthermia are discussed. We also mention in silico as a computational model. Buffer solutions, cell cultures, microfluids, dead tissues and animal models are some of the in vitro media that are discussed in this review paper. In addition, some of the animal models used for hyperthermia will be mentioned.

Keywords: Hyperthermia studies, In vivo models, In vitro models, In silico models, Nanoparticle diffusion media

INTRODUCTION:

Cancer is one of the main causes of death worldwide. According to statistical reports in 2015 about 8.8 million deaths were due to cancer, meaning 1 in every 6 deaths. Based on reports released by the American Cancer Society in 2017, there will be an estimated 1,688,780 new cancer cases diagnosed in the US alone.

One of the newest methods used in the treatment of cancer is hyperthermia. Hyperthermia means a condition in which body temperature rises to a value greater than normal. Some conditions, including illness may result in increased heat gain or heat generation of the body, leading to a subsequent rise in body temperature. Recently scientists have used increases in the temperature of the body to treat cancer. This elevation in temperature causes degradation of cancer cells via hydrolyzing the cell's proteins. This technique is under development and currently in the midst of a clinical trial. Local control of heat is very important in this technique to prevent damage to healthy tissues surrounding the tumor. Magnetic hyperthermia is one of the subsets of hyperthermia that allows proper control of local heat. In this technique, a flow of magnetic nanoparticles is released in the tumor site and at the same time an alternating magnetic field (AMF) is applied to the tumor, a combination that leads to the generation of heat[2,3].

In magnetic hyperthermia the properties of magnetic nanoparticles (MNP), the intensity of the magnetic field, the duration of the hyperthermia process and the features of the targeted cancer tissue all affect the result of therapy. Parameters of magnetic nanoparticles which have been studied up until now are size, size distribution or distribution pattern, concentration of MNPs in tissue and MNP chemical composition which controls magnetic properties such as magnetic moment and magnetic anisotropy[4-7].

MAGNETIC NANOPARTICLES FOR HYPERTHERMIA AND THEIR FEATURES:

The size of MNPs controls their kinetic biodistribution, and previous studies show that optimal biodistribution is achieved with nanoparticles that have diameters of around 20-200 nm. These particles need to go through the vascular network of cancer tissue, and a size greater than 200 nm will result in the particles being extracted from the blood by the spleen. On the other hand, a size smaller than 20 nm will result in the particles being flushed out by the kidneys[8,9]. The surface properties of MNPs determine the stability of these nanoparticles and affect their toxicity and biocompatibility and can even affect the capacity of the nanoparticle to generate heat from the alternating magnetic field (AMF). To improve the stability or biocompatibility of nanoparticles, these particles are coated with ligands such as dextran, cationic liposomes, PVA or lauric acid, resulting in a core-shell particle[6,10,11]. MNPs also need to be able to accumulate in cancer tissue[10]. The hydrophilicity of nanoparticles is a parameter which increases blood circulation time and renders nanoparticles more effective in hyperthermia treatment. Surface modification of nanoparticles especially with hydrophilic polymers such as PEG, PVA, PAA, etc. is a common technique for creating hydrophilic nanoparticles[12]. Also, coating particles with polymers helps the distribution of particles in the environment and controls the cytotoxicity and immunogenicity which may occur with the presence of magnetic nanoparticles in the body[9,13].

Nanoparticles are classified into two categories based on their stimulator, which can be either light or AMF. Important particles that are activated by AMF are iron oxide based materials such as Fe3O4 (magnetite) and γ-Fe2O3 (maghemite). Other metallic nanoparticles include Mn, Fe, Co, Ni, Zn, Gd, Mg and their oxides[10].

The most important subject in magnetic hyperthermia

is the concentration and distribution pattern of magnetic nanoparticles at the tumor site which can ultimately affect the amount of heat generation. Uniform distribution of nanoparticles in cancerous tissue is necessary. Based on the works of Attar et al. inhomogeneity of nanoparticles in the tumor can increase the risk of damage to neighboring healthy tissue, since some nanoparticles particles may reach the healthy tissue and cause undesired temperature elevation[14]. These facts elucidate the importance of microenvironment and its influence on the diffusion of nanoparticles at the cancer site and during the hyperthermia process.

MICROENVIRONMENT FOR THE STUDY OF HYPERTHERMIA:

There are three main methods used for the investigation of the effects of magnetic nanoparticles on hyperthermia: in vivo, in vitro and ex vivo.

Studies that are in vivo are those that are tested on the whole body or in one living organ. These studies can also be done on animal models. Since these studies require the involvement of a living animal, it is important to take animal ethics into account, and no experiment can be carried out without signing required animal ethics protocols. Moreover, in vivo studies are time-consuming and require a lot of money. In some cases, we are unable to study the subject in vivo, for example when measuring bulk tumor temperature in hyperthermia treatment. However, in vivo testing is better suited for observing the overall effects of an experiment on a living subject.

In vitro studies are those which are done in a controlled environment outside of the body in a laboratory that simulates the ECM or organs of living organisms. Accordance between in vivo and in vitro studies is directly related to how well we are able to create conditions mimicking those of a living model. In vitro studies are the first step of a clinical experiment, and obtaining positive results in these studies allows researchers to move on to the next steps of the experiment.

Ex vivo studies are carried out outside of the body, but in a tissue with minimal alteration of natural conditions. Here, controlling conditions is easier and there are no issues regarding animal ethics. The superiority of ex vivo over in vitro is the similarity of its results to in vivo studies, since the organ or sample is a live organ with all of its unique features.

IN VITRO MICROENVIRONMENTS:

Figure 1 illustrates the main approaches used when studying hyperthermia in vitro which includes in vitro cell culture, fluidic media, microfluidic devices, polymeric and hydrogel media and dead tissues.

IN VITRO CELL CULTURE:

Gao et al. investigated the effects of nanoparticle properties in hyperthermia treatment. In this study fibroblast cells of mice (L929) and nasopharyngeal epidermal carcinoma cells (KB) were placed on well-plates for 24hr, and then subjected to magnetic nanoparticles that were coated with hydrophilic polymer. After applying AC magnetic field and heat generation, cell viability and cytotoxicity were investigated. Decreased cell viability directly correlates with effective hyperthermia[15]. In another study by Sonvico et al., HeLa, KB and MCF7 cell viability were investigated[16]. Studying the effect of hyperthermia on just a group of cells could not provide researchers with a good insight regarding the results of hyperthermia in the human body, since cell cultures ignore the possible role of ECM and blood circulation.

IN VITRO FLUIDIC MEDIA

One of the main parameters that affects the amount of generated heat in magnetic hyperthermia is the properties of the microenvironment in which the magnetic nanoparticles are suspended. Researchers usually use solvents as microenvironments for in vitro studies in order to investigate hyperthermia. Fortin et al. studied the effect of the viscosity of this media on hyperthermia.

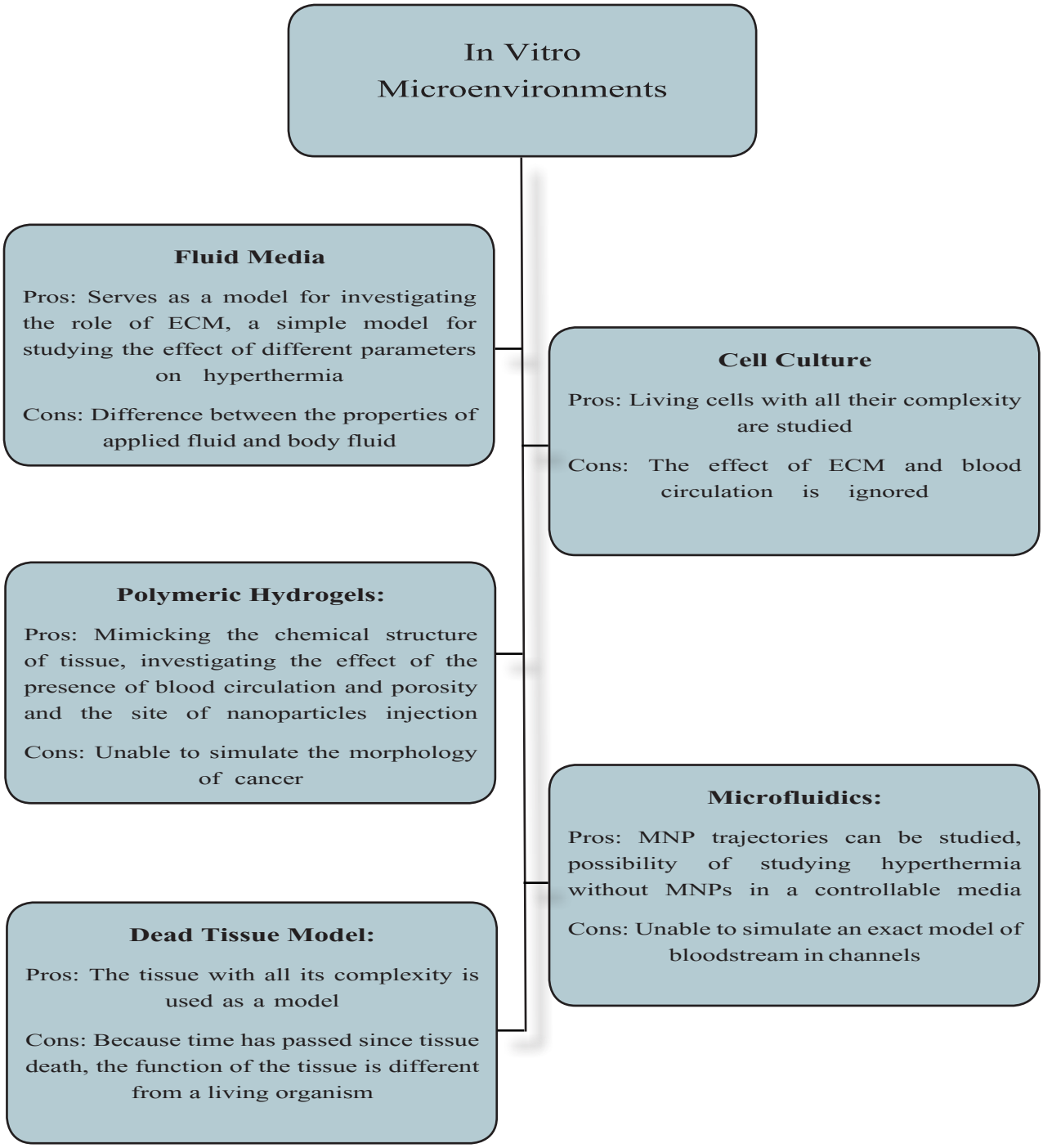

Figure 1. Main In Vitro Models for Studying Hyperthermia.

First, nanoparticles were suspended in an aqueous media and stabilized by electrostatic charges. Afterwards, they changed the media to a more viscous liquid like glycerol, and the amount of generated heat was again measured[17]. Researchers found that there are two mechanisms for heat generation, namely Brownian friction and Néel relaxation. Brownian friction is caused by random and Brownian fluctuations and motions of nanoparticles in a carrier fluid and Néel relaxation is based on internal fluctuations of the magnetic moment of MNPs caused by the AMF. Changing the media to a more viscous fluid increases the effect of Brownian friction. Brownian friction also depends on the nanoparticle's volume. The size of nanoparticles determines which heat generation mechanism is more effective[6,17]. Nemati et al. showed that the main mechanism for heat generation is Brownian friction in small nanoparticles and Néel relaxation in larger nanoparticles. They also studied the effect of nanoparticle shape on heat generation in magnetic hyperthermia. They used water as a media in all of their in vitro experiments[18,19].

Another in vitro study by Murase et al. showed the effect of nanoparticle concentration in a petri dish with saline solution and AC magnetic field on heat generation. They showed that increasing nanoparticle concentration will increase the amount of generated heat[20].

One of the parameters that affect specific absorption rate (SAR) and therefore controlled the amount of generated heat, is the pH of the fluid media. Based on the works of Iglesias et al. the pH of ferrofluid affects SAR and the stability of magnetic nanoparticles in the media. Whenever the pH approaches neutral from either the acidic or alkaline region, SAR and stability decrease. Another criterion which controls SAR and stability is the ionic strength of the media. Researchers added some NaCl to the water that is the media of ferrofluid and observed that a higher NaCl concentration (high ionic strength) results in a decrease in SAR and stability[21]. However, Guibert et al. observed that adding ammonium chloride to water does not affect SAR or stability[22].

Finally, in vitro fluid media which act as ECM in hyperthermia studies is not a proper model for the human body, since the properties of the applied fluid are different from body fluid and this makes the result different from the results of hyperthermia in real tissues.

MICROFLUIDICS AS AN IN VITRO MODEL

Microfluidics are another media for in vitro studies of hyperthermia. As we mentioned earlier diffusion of MNPs in cancer tissue is critical, therefore it is helpful to track particles through the tumor. Bahadorimehr et al. used microfluidics, whose properties could simulate a vascular network in a cancer tumor, in order to track the path of nanoparticle diffusion. They injected MNPs inside microfluidic channels and applied a magnetic force to the system from below. This resulted in the deposit of MNPs on the bottom of these channels, allowing nanoparticle trajectories to be observed[23].

Lin et al. while investigating hyperthermia in vitro, immobilized cells in microfluidic channels and then suspended MNPs in water, injecting the suspension through these channels. AC magnetic field was applied to the system, and cell viability showed successful hyperthermia[12].

Microfluidics can also be used as a media to investigate hyperthermia without nanoparticles. In a project done by Wang et al. direct heat was applied through a microfluidic channel from upstream, and cell viability was evaluated. Upstream where the temperature was about 42°C necrotic death of cells was observed, and throughout the channel with progressive temperature decrease, cells were seen in the late and early stages of death[24].

POLYMERIC HYDROGELS AS IN VITRO MODEL

Researchers are looking for ways to improve in vitro

studies to increase conformity with in vivo results. One way to do so is by using polymeric materials as the media for hyperthermia systems. Alvarez et al. used agarose phantoms as the media for experiments to simulate tissues of the body. In order to make agarose phantoms that mimic healthy tissues they used saline solution, agar, and sucrose, and NaCl, agar and sucrose were used to emulate the cancerous tissue. They used agarose cylindrical phantoms as a model for healthy tissue and the emulated cancerous tissue were placed inside these phantoms, either with or without magnetic nanoparticles. After applying radiofrequency, the amount of heat was measured in both samples[25]. In another study Holligan et al. used agarose gel to simulate the environment of the eye as a media for releasing ferrofluid[26].

The reason for using agar gels as models for healthy or cancerous tissue is the porous structure of gels which can simulate the environment of real tissues. Hard or soft tissues with their unique structures can be simulated by differing gel concentration in the solution. Also by injecting fluid flow to the gel or creating paths through the gel using microtubes (similar to Attar et al. in their study using polyethylene tubes) the effect of blood circulation on hyperthermia can be investigated[14,27]. Salloum et al. used agarose gels as a model for cancerous tissue when investigating heat distribution. There are two methods for delivering nanofluids into tissue. One is injecting nanoparticles intravenous which is at the spotlight of recent researches. Intravenous injection seems to be more efficient and allow the particles to move faster through the tumor and have a better distribution because of the compact vascular network in the cancer tumor. Also, this method gives us a chance to use fewer nanoparticles and decrease the risk of nanoparticle accumulation in body but there is some restriction in this method especially in less porous tumors. In these tumors, in the case of low concentration of nanoparticles the generating heat is few and if the concentration is high, the accumulation will occur and it damages healthy tissue. The other method is the injection of particles strictly into tumor bulk and ECM. Another method is injecting nanoparticles into the extracellular space of cancerous tissue. In this study, researchers used the second method. Results show that whenever the injection flow rate is lower, the distribution pattern of nanoparticles is more similar to a spherical distribution, in which the control of temperature in the tissue is simpler. According to the amount of SAR in various areas of the gel, the concentration of nanoparticles can be measured, showing that concentration is greater at the injection site[28].

Agar gels are another media for in vitro studies, like the one conducted by Lahiri et al. They compared 4% agar gel with water as a fluid media. The level of SAR was measured: for agar gel, it was 91.7 W/gfe and for water, it was 111.8 W/gfe. This means an approximate 40-50% decrease in SAR. This reduction was due to the omission of Brownian relaxation. Agar gels with a concentration greater than 4% can simulate hard tissues, with lower concentrations mimicking soft tissues with the same porosity. Agar gels consist of agarose polymer and agaropectin, a charged polymer with sulfate ions[29].

Generally, the movement of nanoparticles depends on the environment and the size of the particles, both of which affect the concentration of particles in the tissue and heat generation. Kalambur et al. studied the movement of nanoparticles in type I collagen as an in vitro model and used magnetic resonance imaging and infra-red imaging for visualization of the movement. Studies show that the time needed for movement of a group of nanoparticles with a certain number across a 5mm distance in collagen is 100 min, but this same group of particles can traverse a distance of 10mm in glycerol in 10 min (the external magnetic field is

fixed). Also, the amount of generated heat in collagen is much lower than water or glycerol. Researchers link this case to the mechanisms of heat generation and the high viscosity of collagen in comparison with water or glycerol, leading to the prevention of Brownian relaxation and heat generation decrease. On the other hand, collagen has a porous structure that may trap nanoparticles and inhibit their rotation in the magnetic field. Based on previous studies, in nanoparticles coated with polymers or other materials which decrease the interaction of the surface of nanoparticles with the media, Brownian relaxation can lead to loss of energy and a rise in generated heat, similar to how Kalambur et al. a used dextran coating to increase the loss of energy[6].
In silico is another term which is commonly used in hyperthermia studies. Unlike common mentality, in silico studies are performed on a computer or via computer simulation. There are many different computer models for investigating the effect of media on hyperthermia, but we will not address them all here. However, it seems necessary to include an experimental model for a computational study and hint at the methods that can be used. For example, Kalambur et al. used collagen as in vitro base model in mathematical modeling. In computational modeling we need to consider the complexity of the media. For instance, collagen is a very viscoelastic polymer with diverse features, and studying the stress levels in this polymer can be very complex. Therefore researchers, especially in mathematical modeling, tend to use water or glycerol as their microenvironment[6].
Besides the simulation of the chemical structure of real tissue by polymers, which can affect the results of hyperthermia, the effect of the morphological structure is also very important in hyperthermia treatment. Polymers as bulk cannot simulate tumor structure and this leads to errors in results that are obtained from polymeric models.

DEAD TISSUE AS AN IN VITRO MODEL

Due to limitations in the field of study on living organisms, Attar et al. suggested a new method that supersedes dead organs instead of living ones. In this study, flow of blood will go through a death organ in vitro to simulate the performance of a living organ. In hyperthermia the behavior of live and dead tissue are different. This difference is due to the bloodstream that is always present in live tissue. When the balance of input and output of heat in an organ is lost and the temperature increases, the bloodstream will decrease the temperature and create balance again. Whenever the blood velocity and volume are high, the decrease in temperature is greater. If we create a bloodstream in dead tissue similar to that existing in its live counterpart we can study hyperthermia in a condition near to reality. Generally, every liquid with properties similar to blood, such as special heat capacity, conduction, viscosity and density could supersede blood. In this study saline (NaCl in water solution) was selected. Other researchers studying hyperthermia should consider the fact that cancerous tissues are different from normal tissue regarding their vascular network, and the bloodstream is therefore completely different. This difference must be considered in, in vitro studies in dead tissue[7,30].

IN VIVO MODELS

Rat Models

For in vivo studies researchers usually use rat models before confirmation for testing on humans.
Hilger et al. injected magnetic nanoparticles intravenously and let them diffuse into the tumor through the vessels. They observed that the temperature rise was less than what was expected based on in vitro studies with the same type and concentration of nanoparticles and also the same alternating magnetic field properties. This phenomenon was related to the blood circulation

through the tumor in vivo and its acting as a coolant to prevent the temperature rise of the body. On the other hand, the presence of nanoparticles in the body created immunogenicity and antigenicity problems. Researchers observed that formation of localized heat spots is possible in the presence of blood flow through the tumor for in vivo studies. Investigations showed that the temperature difference in tumors is due to their different morphologies, that creates various dispersion patterns for magnetic nanoparticles[31].

In vivo studies should be done with biocompatible nanoparticles. Some particles are intrinsically biocompatible like superparamagnetic iron oxide MNPs, and other particles can become biocompatible with biocompatible coatings such as polymer coats. Without these features, nanoparticles will be ejected from the body by RES uptake from the blood circulation. Researchers should therefore minimize opsonization for MNPs[32]. Another important problem for in vivo studies is the excretion of nanoparticles after hyperthermia treatment. MNPs should be exerted from the body by macrophages or neutral granulocytes and degraded in their liposomes[31].

Temperature mapping in vivo is of great importance in hyperthermia research, especially temperature controls to avoid damage to neighboring healthy tissues. Some common techniques for temperature measurement at the tumor site are 1. magnetically intensive fiber optic probes 2. imaging with the help of superconducting quantum interference sensors 3. MRI thermometry 4. thermochromic fluorescence 5. molecular diffusion by functional MRI and nowadays the use of software such as Nano Plane or Signal Hyper Plan[32].

Examples of In Vivo Studies:

The most commonly used animal models are presented in **Table 1**. Dennis et al. studied the effect of MNPs on breast tumors in vivo. Twelve female mice (C3H/HeJ) with MTG-B murine breast tumor were divided into three groups. After anesthetization by injection of

Table 1. Summary of the main models used for hyperthermia studies.

Hyperthermia Studies	Microenvironment	Culture	Main Results	References
In vitro	Cell culture	Human or mouse cells	Cell viability is measured	(15,16,35)
	Fluid media	Water	Effect of pH, ionic strength and viscosity of fluid is measured	(6,17–22)
		Glycerol		
		Saline solution		
	Microfluidics	Micro fluidic devise	Tracking the path of nanoparticles	(12,23,24)
	Polymeric models	Agarose gels	Mimicking the chemical structure of tissue, the effect of chemical structure is measured	(6,25–29)
		Collagen		
	Dead tissue model		Effect of morphological structure is measured	(7)
In vivo	Rat models	Murine mice	The best similarity with the human body, gives us information for human cancer therapy	(31–34,36–38)
		Xenograft mice		
In silico	Mathematical models	Computer	Hyperthermia process prediction	(39–44)

Ketamine/Xylazine, nanoparticles were administrated to the tumors. Afterwards an alternating magnetic field was applied using a coil. One group of mice was treated without nanoparticles in AMF, another was treated with nanoparticles without a magnetic field, and the last group was treated with nanoparticle administration in AMF. Observations showed that in the last group the tumor was invaded 15 min after reaching a temperature of 40.5%. For temperature mapping, fiber optic probes were deployed.

In magnetic hyperthermia, it is necessary to know whether nanoparticles are immobilized in tumor sites, or whether ferrofluid has movement in the tissue and therefore relaxation losses are present. Researchers used the rat model to study this subject. Dutz et al. injected multicore MNPs into tumors, and based on histological studies it was found that nanoparticles formed spots in the tumor sites from which particles were dispersed homogeneously. Evolution of nanoparticles movement occurred ex vivo and showed the immobilization of MNPs, therefore rotation of particles is prohibited and Brownian losses are excluded. This means nanoparticles are strongly bound to the tissue[34]. Laurent et al. studied the effect of Fe/Fe3O4 core/shell nanoparticles on subcutaneous tumors in murine mouse models (B16-F10). Nanoparticles were injected intratumorally and intravenously, following which results were analyzed. Intratumoral injection is more effective than intravenous injection, and magnetic hyperthermia can occur in a shorter time. Furthermore, intratumoral administration may weaken the mouse melanoma following hyperthermia[32].

In some projects, researchers used xenograft models instead of common mouse models. In these models, human tumor cells were injected subcutaneously into special mice called xenograft mice after being cultured in vitro. Xenograft mice are good models for simulating the human body in vivo since they do not reject human cells. Kossats et al. used this model to investigate magnetic hyperthermia on breast and pancreatic cancer, and based on histological results cell viability and proliferation were reduced[36].

CONCLUSION:

Magnetic hyperthermia is an effective treatment for cancer therapy. Before any experimental tests are carried out in the human body, this treatment needs to pass in vivo testing methods and, before that, in vitro. For in vitro studies, whenever the model is similar to real tissue the results are more reliable. Therefore researchers attempt to create models similar to real tissues in order to yield more accurate results. Among those models which we studied, the dead organ model in the early moments after scarification can be a suitable model for hyperthermia studies. However, each model has its own special features which, depending on the parameters we want to study could be considered an appropriate model.

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