SZIU Faculty of Veterinary Science, Department of Pharmacology and Toxicology

3D tumor cell culture for testing chemicals and treatments: a promising method for future *in-vitro* studies

Author: Jake Oster-Weinberg

Supervisor: Csaba Kovago DVM associate research fellow

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1. List of abbreviations

3D	Three dimensional
BAX	BCL-2-associated X protein
ECM	Extracellular matrix
FADD	Fas-accociated protein with death domain
HT	Hyperthermia
HMGB1	High motility group box 1 protein
Hsp 60	Heat shock protein 60
Hsp 70	Heat shock protein 70
Hsp 90	Heat shock protein 90
IHC	Immuno-histochemistry
MCTS	Multicellular tumor spheroid
mEHT	Modulated electro hyperthermia
NK	Natural killer cell
OMM	Outer mitochondrial membrane
RF	Radio frequency
RTK	Tyrosine kinase receptor

2. Introduction

Cancer is now one of the most prevalent diseases in today's society. The rate of cancer has grown so greatly it is now estimated (a study conducted by cancer research into the risk of being diagnosed with cancers excluding non-melanoma skin cancer) that one in every two people will be affected by cancer in their lifetime. This undoubtedly highlights the importance of refining and perfecting treatments. In the veterinary profession, in particular with pet animals as the number of animals being diagnosed with tumors is steadily increasing. Heat treatment generally, is a particular form of treatment often in combination with radiotherapy or chemotherapy, with many reporting's of positive prognosis after the combined treatment. It is the hyperthermia alone that has not been the main focus of research and refinement of the technique.

Modulated electro-hyperthermia (mEHT), also known as oncothermia, is used in 15 countries around the world, and by more than 180 institutions. More than 100,000 mEHT treatments are conducted yearly and have delivered remarkable increases in life expectancy and quality of life. Success rates in clinical trials show values of up to 80% having a positive response to the treatment. Nowadays this method is being introduced to the veterinary therapy as well, currently as experimental treatment or as clinical trials. It has been proven that there is a direct correlation with remission of the disease, be it partial or full, with a negative correlation of the disease progressing and continuing its malignancy. The problem that arises comes from this treatment is the lower consistency of results obtained in *in-vivo* experiments. There are a smaller proportion of results obtained which follow the trend in in-vitro sampling.

Animal subjects are widely used in biological research. However, due to growing animal welfare issues and backing of animal rights campaigners against these kind of experiments, there has been a limit to the number of animals used and the methods of experimentation. There are now requirements to reduce the number of animals used, requirements to refine the experiments to refine the testing that is performed in order to yield more data and to ensure more accurate data, and lastly to replace the living animals as much as possible. This three "R" rule has led to development of new experimental models. One kind of these new models is the multilayer cell culture system, also called as three dimensional (3D) cell cultures. These models have a higher resemblance to *in-vivo* systems

than the commonly used monolayer cultures. The interconnections between the cells and the relatively high amount of extracellular matrix (ECM) in these cultures make them capable of accurately stimulating conditions which are present in living animals. Efforts to refine and develop these kind of model systems can result in animal experiments being reliably performed using *in-vitro* 3D cell cultures in the future.

3. Scientific goals

Our aim of the experiment has three main goals to achieve. Our preliminary aim was to create and utilise a stable 3D prototype which reflects *in-vivo* tissue. This prototype must be reflective in its structural properties as well as its response to treatment. This will be tested by heating, namely in the form of regular hyperthermia and modulated electro hyperthermia, and its results evaluated. We were also intended compare the results to previous in-vitro experiments data. If we can analyse the effects on a cellular scale, we can harness its effects to make treatments more effective in future. We want to see if the results are in conjunction with *in-vivo* methods and whether the in-vitro experiments can eventually replace *in-vivo* testing.

4. Literature review

It has been known for many years the effect of heat on cells, particularly on cancerous cells and their ability to withstand a variety of environments differing from the normal. Hyperthermia is referred to in oncology as the treatment of malignant diseases by administering heat in various ways. In our experiment the culture is heated and controlled by a single thermodynamic parameter. Oncothermia is a subgroup of hyperthermia, where the heat increase is in direct relation to a dosage of radio-electric energy radiation. It is able to deliver directed heat to multiple lesions throughout the entirety of the malignant cells, while leaving the healthy cells unaffected (Hegyi et al., 2013). Oncothermia has been used practically since 1989 and while its clinical results show positive results the precise mechanism is still unclear even today (Andocs et al., 2009).

Both hyperthermia and oncothermia were used as a form of treatment for the cell cultures. The initial use of using hyperthermia as a form of treatment dates back to 1898, however there has been doubt to the performance due to complications of directing the heat and ensuring no complications in the deep heating (Hegyi et al., 2013). Since then the process has been refined to reduce the detrimental effects to healthy cells as well as beneficial effects to the tumor (Yanase et al., 1998; Matsuoka et al., 2004). The basic notion of hyperthermia is to achieve a temperature above 37.5°C, with a defined temperature between 41 and 47°C. This technique can be used independently or in conjunction to alternative therapies, namely chemotherapy and radiotherapy. It has been accepted that hyperthermia is not a solely sufficient method to combat tumors, however it is undisputed the synergistic effects when combined with cytotoxic drugs or radiation. It has been recorded clinically that patients being treated with radiotherapy and hyperthermia as opposed to radiotherapy alone showed higher rates of survival and an increased response to the treatment. However, solely hyperthermia at lower temperatures such as 43°C have been proven to show tumor regression with repeated treatment. There is evidence that hyperthermia treatment will result in an immune response against the tumor causing further positive prognosis and tumor regression (Hildebrandt et al., 2002; Garcia et al., 2012).

Despite its positive results hyperthermia does have its limitations as a form of treatment clinically. Its use must be restricted in certain treatments namely in lung tumors, as there is a variable chance to exaggerate and aggravate preexisting pleural fluids. On top of

this, due to the physical movement of the thorax, focusing the heat to maintain a desired temperature has proven ineffective (Szasz, 2014).

When hyperthermia treatment can be applied it works by causing the cells to perform apoptosis. The cells' apoptotic response to hyperthermia is in conjunction with the cells' mitotic phase. Cells undergoing heat treatment in the M-phase reveal damage to their mitotic infrastructure, leading to abnormal mitosis and resulting in polyploidy cells. Cells in their S phase demonstrate chromosomal damage. The M and S-phase are able to undergo a slow cell death. Cells in their G1 phase are able to resist damage by heat treatment, and do not show noticeable chromosomal damage, although they will show a rapid form of cell death post hyperthermia (Hildebrandt et al., 2002).

Apoptosis is performed through one of its two pathways, the intrinsic pathway or the intrinsic otherwise known as the death domain pathway. Both these pathways result in the cleavage of the caspase-3 protein which will then go on to perform the lytic actions on the apoptotic cell: DNA fragmentation as well as cytoskeletal and protein degradation. Apoptotic bundles will form which can eventually be removed by the action of phagocytic cells (Elmore, 2007). In order to determine the prevalence of apoptosis, TUNEL assay (explained in detail later) and BAX antibody staining is often used to visualise apoptotic signs especially apoptotic nuclei.

Apoptosis occurs through a mechanism using different caspases, a variety of initiator and executioner caspases. Initiator caspases such as caspase-8 and 9 are activated by dimerisation through FADD, being activated by a ligand-death receptor complex. This dimerisation causes a cascade of reactions resulting in activation. These initiator caspases can then activate the inactive procaspases, namely 3, 6 and 7. These three executioner proteins will cause the resulting apoptosis of the cell. Caspase-3 will result in direct and non direct apoptosis. It enables activation of further caspases, namely 8 and 9 which will cause death substrates being cleaved along with a loss of cell viability. Caspase-3 will directly cause a condensation of chromatin as well as DNA fragmentation and Blebbing, through DFF-45 and alpha-fodrin (Porter and Janicke, 1999). Caspase-3 is a frequently activated death protease in apoptosis. It is noteworthy that caspase-3 is important for survival, enables regular morphology and viability. It has been shown that caspase-3- deficient mice were born at

lower frequency and survival is limited to a few weeks (Kuida et al., 1996). Apoptosis is not limited to hyperthermia and will also occur due to oncothermia by the same protocol.

As well as hyperthermia making the cell undergo apoptosis, it is also able to alter the cell's structurally integrity. Not only is it able to apoptise cells but it can remold and reshuffle already present structures necessary for viability and structural grounding. For longevity of tumor cells its ability to block, and alter angiogenisis is of vital importance. This will reduce oxygen and nutrient supply as well as causing intratumoral acidosis. This is in conjunction with the simultaneous effects of hyperthermia, such as alteration in blood cell membrane, microthrombus formation and a shift in the plasma fluid into the interstitium, creates an environment unfavorable to cell survival and encourages the death of the cells (Shimizu et al., 1996; Hildebrandt et al., 2002).

It has been demonstrated that hyperthermia has been proven to inhibit angiogenisis, resulting is less growth and viability of the tumor, lowering its chance of survival and metastasis. In addition to this, hyperthermia in tumor cultures can induce an intracellular and extracellular pH change. This in turn will sensitise the tumor cells to hyperthermia resulting in a positive feedback loop. Its mechanism is as follows: an increased glycolytic rate with accumulation of lactic acid, an intensified ATP hydrolysis, an increased of ketogenesis with accumulation of acetoacetic acid and B-hydroxybutyric acid, an increase in CO_2 partial pressures, changes in the chemical equilibrium of the intra- and extracellular buffer systems, and an inhibition of the Na^+/H^+ antiport channel in the cell membrane (Vaupel and Kelleher, 1995).

The main drawback of hyperthermia is its lack of selectivity of the cells it affects. While previous results have been proven effective, it induces damage into healthy and non malignant cells causing widespread apoptosis. Due to this less controlled mechanism of action, other more selective methods are preferred, in particular oncothermia (Hegyi et al., 2013).

4.1. Modulated electro-hyperthermia

Cancerous tissue is different from healthy in a variety of ways. There is one specific difference which can be exploited to focus and direct heat and radiofrequencies, that is the higher metabolic activity of cancerous cells and their ability to absorb the alternating current at a different rate to healthy tissue. Modulated electro-hyperthermia works by an amplitudo-modulated radiofrequency (RF) signal of 13.56MHz being applied which is absorbed in the body primarily in the membrane and extracellular electrolytes.

Cancerous cells have been shown to have an increased metabolic activity. Due to this they posses a higher dielectric constant of the extracellular matrix resulting in higher conductivity than normal tissues. (Fiorentini and Szasz, 2006). This causes the RF current to automatically refocus by the lower impedance, enabling the current to flow in the extracellular space. When this electric field is maintained, it causes temperature gradients of 1 K/mm, enabling a heat flow of 1500nW/mm2, (roughly seventy times higher than basal levels) into the cell. In conjunction with this, there is a simultaneous ATP dependant sodium influx which also results in heat production. A higher permeability for water than ions will result in an increase in intracellular pressure. Malignant cells have a higher phospholipid concentration in their membranes resulting in a more rigid membrane. The membranes of these cells are less elastic and more sensitive to pressure changes, and therefore are more susceptible to the treatment (Hegyi et al., 2013).

While oncothermia has many advantages over simple hyperthermia, the fact that the heat flow in the cytosol of cells is continuous until an equilibrium has been met is of greater importance as it increases its reliability and its efficiency. mEHT is not limited by the membrane thermal limit and is able to effect specific groups of cells among healthy cells (Hegyi et al., 2013). mEHT and hyperthermia differ greatly in the treatment of tumors in particular in the possibility of forming metastatic tumors. Hyperthermia has a slight tendency to promote dissemination of malignant cells (Hegewisch-Becker et al., 2003). When tumors become metastatic, through the lymph or blood, the cancer becomes much more severe and there is a greater chance of a life threatening disorder. On the other hand mEHT blocks the dissemination, avoiding the mobility due to enhancing the connections between the tumor

cells. This aids adhesiveness and restricts the individuality, a characteristic of malignant cells. (Hegyi et al., 2013).

4.2.3D cell cultures

Cell cultures have been widely used to mimic conditions of tissues *in-vivo*. 2D cell cultures while offering a sufficient level of similarity to living tissues, are limited in their function. Despite their large use in comprehending cell cultures, they are restricted. Cell adhesion, structure and response to stimuli are vastly different in 2D and 3D cultures (Weiswald et al., 2015). The differences in cellular function allows further and more detailed analysis compared to its 2D counterpart. Many cells isolated from tissues and inoculated on 2D cultures, result in a flat structure and can lose their phenotype. It has been noted that some of these cell types can return to their physiological form and function when placed in 3D cell cultures (Benya and Shaffer, 1982; Baker and Chen, 2012). This of course highlighting the importance of witnessing the definitive outcome of cells to external conditions.

In order to create 3D tumor cell cultures, there are various models which can be used. These models are determined by the setup, which can be multicellular tumor spheroid models (MCTS), organotypic slices of cancer tissue, multilayered cell cultures and using scaffolds (Weiswald et al., 2015). These spheroids can be grown initially from two main sources. Established cell lines as well as directly from primary tumors. The growth of tumors *in-vitro* has comparable features to that in live cells. As the tumor grows, the proportion of proliferating cells decreases where as the quiescent cells increases. If the cells are in suboptimal conditions, (namely glucose and oxygen) cell death and necrosis can be observed in the center, amplifying the importance of these parameters, in particular when different cell layers in a 3D format are needed (Sutherland, 1988).

Tissue-derived tumor spheres were, initially used for *in-vitro*, human colon cancer cell lines. This resulted in tissue derived tumor spheres being predominantly used in colorectal cancer (Mcbain et al., 1984).

MCTS can be easily generated by several methods, one of which is the liquid overlay technique. Tumor cells are placed on a tissue culture which is covered by a thin layer of agar.

The film is dried and then a medium is added enabling cancer cells to grow, only adhering to other cancerous cells (Yuhas et al., 1977).

The hanging drop method is another viable method for producing MCTS. Cell droplets are placed on the underside of a culture lid. Once the lid is inverted MCTS can form due to the gravitational forces and adhesive properties of the cell culture. This method avoids using coating substances. It is worth noting that tumor cells have characteristically, a lower adhesive character. There is a lack of inhibition contact factor which will result in tumor growth, the tumors invasive properties and its ability to metastasize, therefore this method is not very regularly used (Abuelba et al., 2015).

The most recent and possibly the most credible method for MCTS production is by using microcapsules with alginate-based membranes. Cancerous cells are encapsulated in cellulose based microparticles. These cells have narrow size distribution, due to a peroxidase-catalysed reaction in water-immiscible fluid under laminar flow. The microparticles are then covered with an alginate-based gel. Here the cellulose based microparticles undergo degradation by cellulase. This method is able to form MCTS from cancer cell lines which are unable to be produced by the previous two methods stated above. The drawbacks of this method is that the alginate membrane reduces oxygen and nutrient supply as well as cell contact.

4.3. Protein matrix gel scaffold technique

Protein hydrogels have been increasingly used in tissue engineering due to the strong similarities to tissue in its natural form, with regards to viability, strength and composition, while simultaneously being able to create a microenvironment. The scaffolds are created in a similar manner to the ECM. Proteins such as collagen, laminin and fibronectin are commonly used as well as proteoglycans to ensure maximum viability (El-Sherbiny and Yacoub, 2013; Lu et al., 2013).

Cellular adhesion and communication is predominately by desmones. It has been considered that the inability of coupling between cells is a greatly important factor in tumor

proliferation. It is noteworthy that the permeability and structural integrity of the membranes are not altered during tumor growth (Sutherland, 1988).

4.4. Heat shock proteins

There is a broad spectrum of heat shock proteins (Hsp), however Hsp60, 70, and 90 are of particular importance. Hsp60 plays a central mitochondrial role in the folding mechanism of the matrix. Hsp60 is regulated by cochaperonin Hsp10. The formation of mitochondrial proteins is impaired by mutant temperature dependant Hsp60, which will in turn act by inhibiting normal polypeptide chain folding. At raised temperatures, Hsp60 is required to maintain its active state, causing persistant misfolding (Gething, 1997).

As well as Hsp60, Hsp70 is also associated with protein formation and folding in the cell. Unlike Hsp60 there are no transduction pathways, instead acts by checking the structure and ensure correct alignment. It does so by enhancing folding, solubilisation and refolding of aggregated proteins and aims prevent aggregation in alliance with its JDP co-chaperone. The activity of this is maintained by Hsp70's affinity to hydrophobic protein segment in an ATP dependant manner (Gething, 1997). It has also been demonstrated that HSP70 has a key role in immunity mediated by antigen presenting cells in conjunction with T cells and natural killer cells. Hsps originating from tumors are able to transport immunogenic peptides towards MHC I, initiating its associated pathway. Natural killer (NK) cells c-type receptor CD94 are able to bind to HSP70 on a targeted tumor cell which will initiate the production of apoptotic inducing enzyme, granzyme B. Once this granzyme is released it binds to Hsp70 forming an artificial channel in the membrane, aiding internalization and initiating apoptosis (Radons and Multhoff, 2005).

As well as the previous two heat shock proteins, Hsp90 is also of great importance, (and has been argued that it is more than the previous two heat shock proteins) specifically in its protooncotic effect. Hsp90 helps the folding and proteolytic turnover inside the cell. Increased expression of Hsp90 aids survival in cells under undesirable condition, such as heat, hypoxia or acidosis, these conditions being prevalent in tumor cells. It enables tumor cells to become tolerant of mutations to signaling molecules which otherwise would be lethal. One can think of tumor cells as having a high affinity for Hsp90, aiding cell survival. Several

agents such as 17-allylamino are being developed to combat these problems with cancer treatment (Bagatell and Whitesell, 2004). A major group of Hsp90 proteins in the tyrosine kinase receptor (RTK), abundantly seen in cell motility. When these cells are in the presence of chemo attractants, the RTKis activated and stimulates cell motility, ie, the ability to metastatise (Tsutsumi et al., 2009).

In conjunction with the above, Hsp levels are increased in cancerous tissues, and often the levels of certain Hsps can be used as a prognosis for recovery: an over expression being associated with poor prognosis. The elevated levels result in the cells defending against cell signaled apoptosis, and aids viability of the tumor. It has also been suggested that Hsps are stimulated by an increased in denatured proteins, preventing full apoptosis (Bagatell and Whitesell, 2004; Ciocca and Calderwood, 2005).

In conjunction with the intracellular Hsps, they have also been observed extracellulary as well as on the plasma membrane in cancerous cells. Hsp70 and 90 can be used as stimulants for the adaptive immunity. Hsp-chaperone complexes are taken up by antigen presenting cells and can be presented for cytotoxic T cells on MHC I molecules (Suto and Srivastava, 1995).

4.5.HMGB1 protein functions

High motility group protein 1 (HMGB1), a nuclear protein active both intracellular and extracellular, potentiating and mediating the innate immune response, contributing to its proinflammatory role externally and acting as a chaperone protein for DNA intracellulary (Kang et al., 2013). HMGB1 is released as a result of cell damage or necrosis. The release of this protein is significantly reduced in apoptotic cells, however, due to engulfment by macrophages (of which HMGB1 is also present) it may similarly induce HMGB1 levels (Yang et al., 2013).

In accordance to the cellular functions, HMGB1 plays vital roles in the nuclear function of the cell. HMGB1 is stored in the nucleus where it is stores in the A or B box. HMGB1 can be activated into the cytosol by hyperacylation through p53 transactivation, TLR4 binding or by RAGE binding domain. These boxes enable binding to DNA structures,

causing nuclear homeostasis and stability of the genome. Nuclear HMGB1 acts on the nucleosome structure by promoting nucleosome sliding and increasing the ability of DNA to bend. It aids gene transcription by increasing the sequence specific transcription factors. Losses of HMGB1 have proven to decrease DNA repair efficiency and increase DNA damage (Kang et al., 2013).

The coinciding oncotic and anti tumoral effects of HMGB1 can be seen at parallel times. The protein can be split into intra from extra HMGB1. The extracellular promotes the pro oncotic functions which were stated before, however also plays a role in the immunogenic cell death of cancer cells, as well as stimulating immunity during cancer treatments, namely chemo and radiotherapy. Intracellular HMGB1 acts positively by promoting autophagy of tumoral cells but simultaneously can promote tumorgenisis (Kang et al., 2013).

There are however complications of over expression and alteration in the location of the HMGB1 protein. These factors are in accordance with the traits of tumors, namely: unrestricted replication, inhibition of apoptosis, resistance to growth inhibitors and angiogenisis through binding to RAGE, activating the NF-kb pathway resulting in proangiogenic factors. The classical pro tumoral traits of the extracellular HMGB1 result in a positive balance towards a pro oncotic effect, while being balanced by the antitumoral intracellular autophagy response (Tang et al., 2010; Kang et al., 2013).

Normal levels of HGMB1 protects cells against induced apoptosis, be it by casp-8 or Bax induced, where as a down regulation of the gene has been proven to stimulate apoptosis in LNCaP prostate cancer cells through the caspase-3 pathway (Brezniceanu et al., 2003; Gnanasekar et al., 2009).

4.6. BAX protein function in apoptosis

BAX (BCL-2-associated X protein) protein is a member of the BCL-2 family. The members of this family play pro- and anti-apoptotic role in the intrinsic pathway. While for example, BLC-2 has anti apoptotic function, BAX is a strong pro-apoptotic protein. Normally BAX is located in the cytosol. After cellular stress, it is activated via several steps including conformational changes followed by oligomerization of multiple BAX units. The formed BAX oligomer is inserted into the outer mitochondrial membrane (OMM), causing pore formation and these pores are large enough for the majority of soluble mitochondrial internal membrane space proteins to escape into the cytosol. However, the interplay between the BCL-2 family and mitochondria is not limited to the intrinsic pathway (Renault and Chipuk, 2014)

4.7. TUNEL assay

Tunnel assay is a method used to determine apoptotic levels. As apoptosis occurs, DNA is degraded by endonucleases producing double stranded oligonucleosomal fragments, a characteristic of apoptosis. As this occurs deoxynucleotidyl transferase dUTP nick end labels will identify these breaks in DNA by allowing binding through template independent nucleotide addition to 3'OH terminals on cleaved DNA. It is note worthy that as well as apoptosis, tunnel assay can also be used in necrotic cells where karyolysis will also occur, resulting in DNA damage. Due to this it is often regarded as valuable to proceed with further examination, through immunohistochemical staining or western blots. (Loo, 2011; Kyrylkova et al., 2012).

4.8. Effects of mEHT treatment *in-vivo*

Subsequent experiments have been performed to investigate the effect of the mEHT treatment in case of tumor allografts implanted to BALB-C mice. Results showed that mEHT treatment activates Hsp70 heat shock protein in mice. This treatment similarly induces apoptosis locally in the treated tumor in short period after the treatment. This was proved by strong TUNEL positivity and the changed morphology of the HMGB1 protein in the cells. (Kővágó et al., 2014).

5. Materials and methods

5.1. Cell culturing

The C26 cell line was used for the experiment, a carcinoma cell line of murine origin (kind gift from the Semmelweis University). The cells were seeded in a 75 square cm flask in a 10% fetal bovine serum containing RPMI 1640 + Glutamax medium (Thermo Fisher Scientific, Waltham, MA, USA), incubation time was 3 days.

5.2. 3D cell culture preparation

In order to apply the cells to the ECM gel, the flasks were tripsinised for 15 minutes and the cells were recovered in full medium. The cells were washed in 10 ml of full medium, and centrifuged at 1000g for 10 minutes. Finally, the cells were resuspended in the full medium and the final concentration are set to $5.83*10^6$/ml. To create the 3D gel scaffold, 1.4 ml of ECM Gel originating from Engelbreth-Holm-Swarm murine sarcoma (Sigma-Aldrich Inc, St. Louis, Mi, USA) is mixed with 0.6 ml of cell suspension and the mixture was casted to single chamber SlideFlask units (Thermo Fisher Scientific, Waltham, MA, USA), with a gel layer of approximately 2 mm thick. For the experiment 9 of these SlideFlask chambers were made. Once the gel had been left to solidify, the units were put into an incubator under 5% CO_2 concentration and 37°C according to the manufacturer's instructions. After 30 minutes, the gel solidified and the units were filled up with 5 ml of medium mentioned above to provide enough nutrients to sustain the cells.

5.3. Test setup

The units were incubated for 3 days and after that the experiment was carried out. As for the experimental groups setup, four specimen groups were formed, as follows:

1. Control (C): samples were kept at 37°C outside of the incubator.
2. Hyperthermia (HT)
3. Modulated electro-hyperthermia treatment with contact electrode (CO-mEHT)
4. Modulated electro-hyperthermia treatment with non-contact electrode (nCO-mEHT)

Each group consisted of 3 samples, each treatment lasted for thirty minutes.

5.3.1. Hyperthermia group

The hyperthermia experiment focused on heating up the cells at 42oC at a constant rate for 30 minutes on a simple hot plate. (MS-H-Pro, Scilogex LLC, Rocky Hill, CT, USA). All three of the plates were applied to the hot plate at the same time on the same hot plate.

5.3.2. CO-mEHT group

The oncothermia has a more technical setup. Using the Lab-Ehy 100 (Oncotherm Inc., Paty, Hungary) modulated electro-hyperthermia generator, we were able to transmit a modulated radiofrequency current of 13.56MHz through the samples using a specific applicator unit provided by the manufacturer. The applicator has a platinum electrode that transfers the RF energy to the sample. To ensure the direct contact with the gel scaffold, this platimun electrode has to be slightly bent. The temperature of the samples during the treatment was monitored by a Luxtron m600 fluoro-optic thermometer unit (LumaSense Technologies Inc, Santa Clara, CA, USA) with 4 fiber optic sensors which are placed into 4 of the possible 6 locations after a sterilizing spray was applied. The RF power of approximately 12 watts was applied to each of the three oncothermia cultures to ensure a constant sample temperature of 42°C. The treatment setup can be seen in Figure 1. The temperature of the culture is recorded by a fiber optic probe with exposed ends which is cautiously dipped into the medium. Each culture was submitted to the treatment for 30 minutes. (Figure 1)

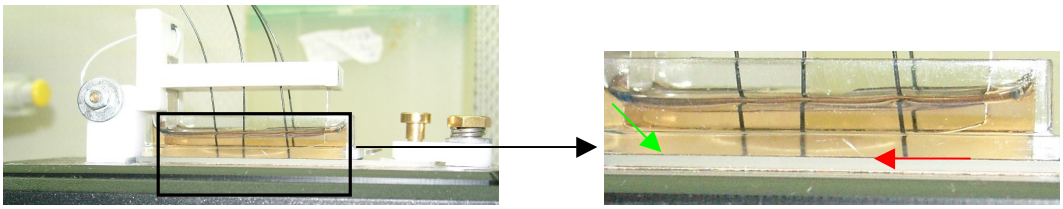

Figure 1.: The images are showing the test setup of the CO-mEHT samples in the applicator for mEHT treatment. The orange fluid is the cell culture medium, the opalic layer (green arrow) is the gel layer containing the cells, the black lines are the thermometer sensor probes and the metalic strip (red arrow) is the treatment platinum electrode, the source of the modulated RF current. The platinum electrode is pushed against/in the gel layer to ensure the direct electrical contact.

5.3.3. nCO-mEHT group

Using the same technical setup as the contact oncothermia, the platinum electrode has been left straight horizontal, although still submerged in the medium it has no direct contact with the scaffold. The temperature measurement and power settings were identical to the previous group's settings. The treatment setup can be seen in Figure 2.

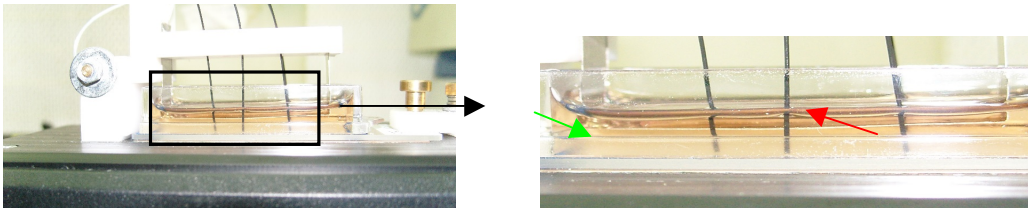

Figure 2.: The images are showing the test setup of the nCO-mEHT samples in the applicator for mEHT treatment. The orange fluid is the cell culture medium, the opalic layer (green arrow) is the gel layer containing the cells, the black lines are the thermometer sensor probes and the metalic strip (red arrow) is the treatment platinum electrode, the source of the modulated RF current. The gap between the gel layer and the platinum electrode is well visible.

5.4. Histology

After the treatment, a 24 hour incubation period was maintained with the samples then placed into 10% formaldehyde solution in order to prepare the histological slides. The gel layer was removed from the samples and was embedded in paraffin blocks. Once the slides have been made, they were stained with Hematoxilin-Eosine (HE). The process has been carried out at the Department of Pathology of the University. The slides have been scanned using the Pannoramic SCAN slide scanner (3DHisTech, Budapest, Hungary) and the scanned data were used for further analysis.

5.5. Immune-histochemistry

In order to identify the levels and morphology of different proteins related to apoptotic signs, immuno-histochemistry stainings were performed on the samples. The stainings were performed in the laboratory of the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University (Budapest, Hungary), using protocol described earlier (Andocs et al., 2015). Briefly, unstained complete cross-section histology slides were dewaxed and rehydrated. After this, the target antigens were retrieved by using heat treatment in Tris-EDTA (pH 9.0) or sodium-citrate buffer (ph 6.0). Following the retrieval the slides were blocked using 5 % bovine serum albumin (BSA)–azide (Sigma-Aldrich, St. Luis, MO, USA) and the primary antibodies were applied to the slides (Table 1.)

Table 1: Summary of the primary antibodies used to stain the samples

Antibody	Working dilution	Manufacturer
Hsp 70 (HSP70 Rabbit Ab, 4872S)	1:50	Cell Signaling
Hsp 60 (HSP60 (D307) Rabbit Ab, 4870S)	1:100	Cell Signaling
HMGB1 (D3E5) Rabbit mAb #6893	1:200	Cell Signaling
BAX (Anti-BAX, HPA027878)	1:50	Sigma-Aldrich

As for secondary antibody, Alexa Fluor 546 (orange-red) coupled anti-rabbit IgG (Invitrogen,) was used in 1:200 dilution. Nucleus contrast staining has been done using 4′,6-diamidino-2-phenylindole (DAPI). The slides have been scanned using the Pannoramic SCAN slide scanner (3DHisTech, Budapest, Hungary) and the scanned data were used for further analysis.

5.6. TUNEL assay

In order to identify apoptotic cells in the slides TUNEL assay was performed as assay was performed in the laboratory of the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University (Budapest, Hungary), described earlier (Meggyeshazi et al., 2014). Briefly, Click-iT TUNEL Alexa Fluor 488 Imaging Assay (Invitrogen, Carlsbad, CA, USA) was used on dewaxed and rehydrated whole cross section slides. Nucleus contrast staining has been done using DAPI. The slides have been scanned using the Pannoramic SCAN slide scanner (3DHisTech, Budapest, Hungary) and the scanned data were used for further analysis.

6. Results

6.1. Native structure of the 3D culture

In Figure 2 we are demonstrating the cell organization of the 3D gel scaffold C26 cell culture we used in our experiments. The image has been taken from the upper 1/3 area of the 2 mm thick culture, and showing the typical nest-like organization of the tumor cells, the nests are connected to each other via cellular bridges in all the three dimensions. Deeper layers show similar picture, although the cell-bridge contacts in the deep parts of the culture are diminishing and the size of the cell groups is getting smaller as we go deeper towards the bottom of the unit.

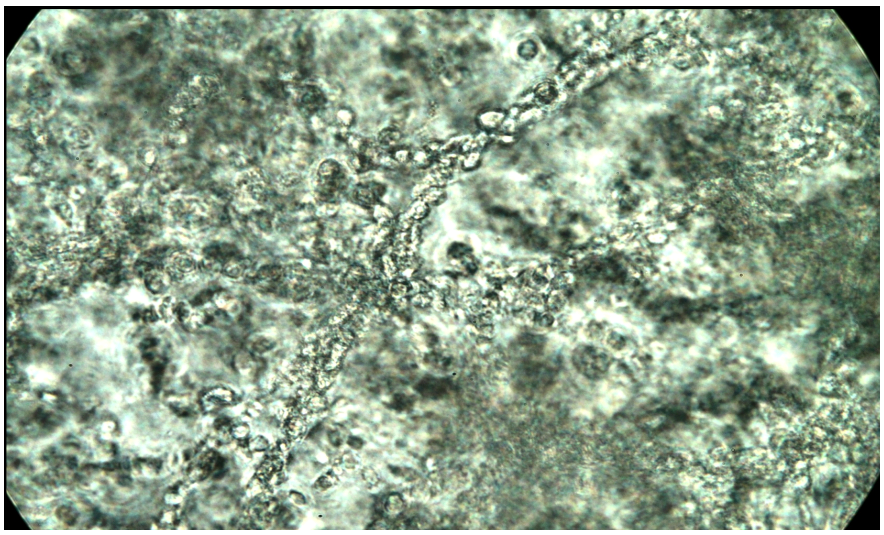

Figure 2: Image of the living 3D C26 3D cell culture. It is well visible, that the cell groups are growing in three dimension and between the cell nests cell-bridges are forming the connection. The magnification of the image is 400x.

6.2. Histology

Cell cultures were fixed in formalin then examined with simple HE staining. We can see differentiation in the results of the treated and control samples. The control slides showed regular cell structure, however, when compared to the treated samples apoptotic bodies were seen as well as a higher amount of pyknotic nuclei indicating some form of damage to the cell, eventually leading to nuclear dissolution (Figure 3.).

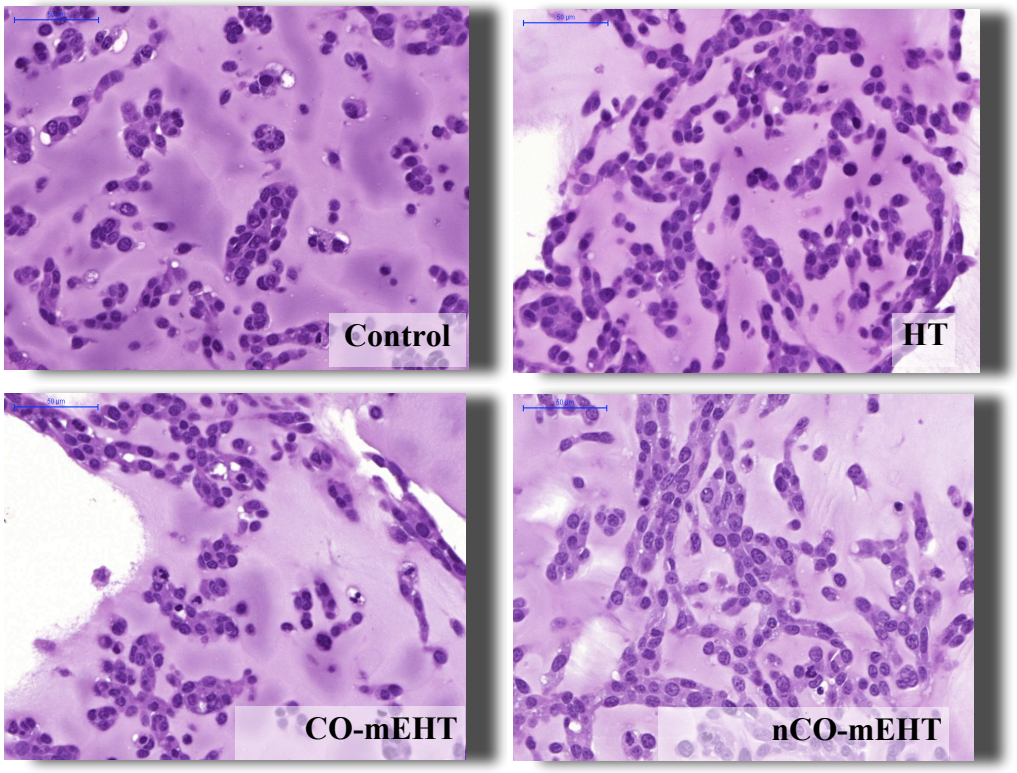

Figure 3.: HE stained slides. Arrows are indicating the small, dense, pyknotic or fragmented nuclei in the tissue. These damaged nuclei were seen in larger numbers in the treated samples, the distribution of them was nearly equal, although in the CO-mEHT samples some local accumulation were observed. Magnification is indicated in the scale bar in each picture.

6.3.Immuno-histochemistry

6.3.1. Hsp60 protein staining

IHC staining resulted in slight differences in Hsp 60 levels in both the control and treated samples. Even, the experienced Hsp 60 positivity in each sample showed up generally throughout the slide (Figure 4.).

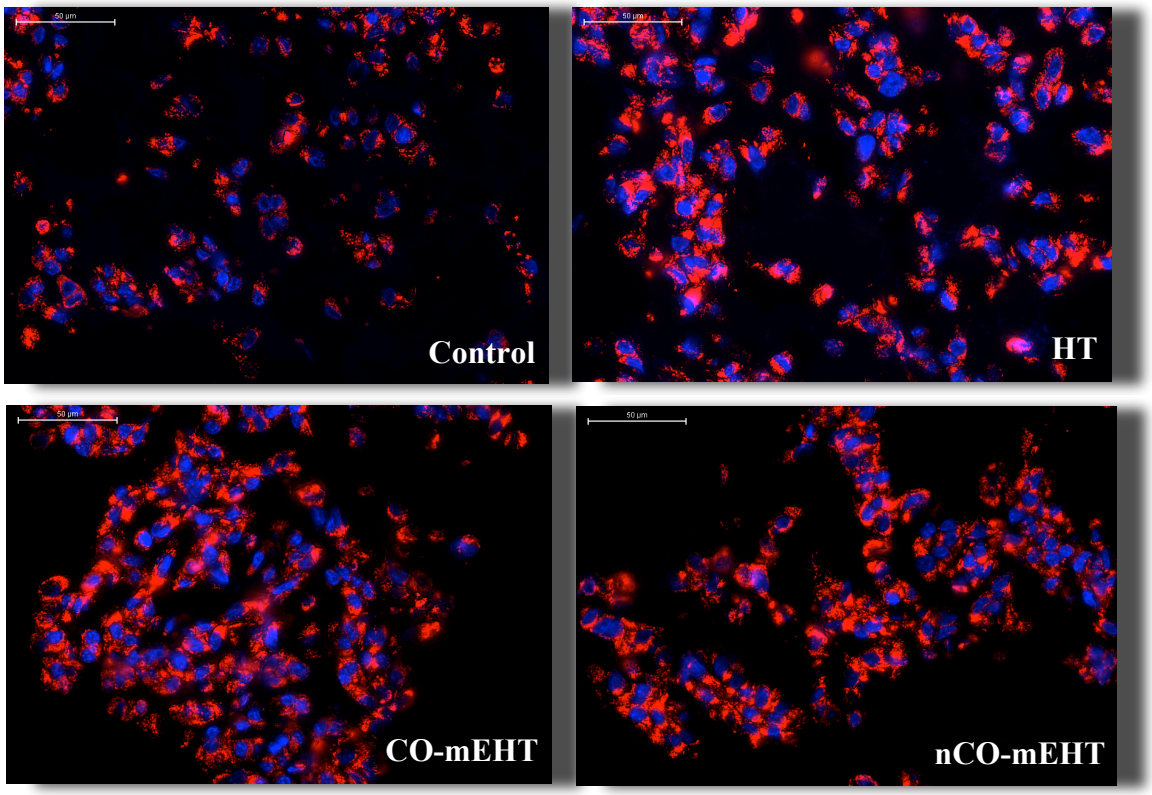

Figure 4.: Images of the results of Hsp60 staining. There is a clear increase of Hsp60 positivity in the heat treated samples compared to the control. Both Co-mEHT and nCO-mEHT show even more raised levels of the protein compared with the HT sample. Magnification is indicated in the scale bar in each picture.

6.3.2. Hsp70 protein staining

Hsp 70 positivity was seen in the treated (HT; CO-mEHT; nCO-mEHT) groups in variable levels. In the HT samples, there was moderate positivity, equal in all the area of the samples as is visible in Figure 5. In the case of CO-mEHT slides, very strong positive staining areas have been seen, whereas other areas of the slide showed low to minimal positivity. In the samples of nCO-mEHT group, moderate to strong positivity were seen in the slides, but the staining was regular in it appearance throughout the slide (Figure 5.).

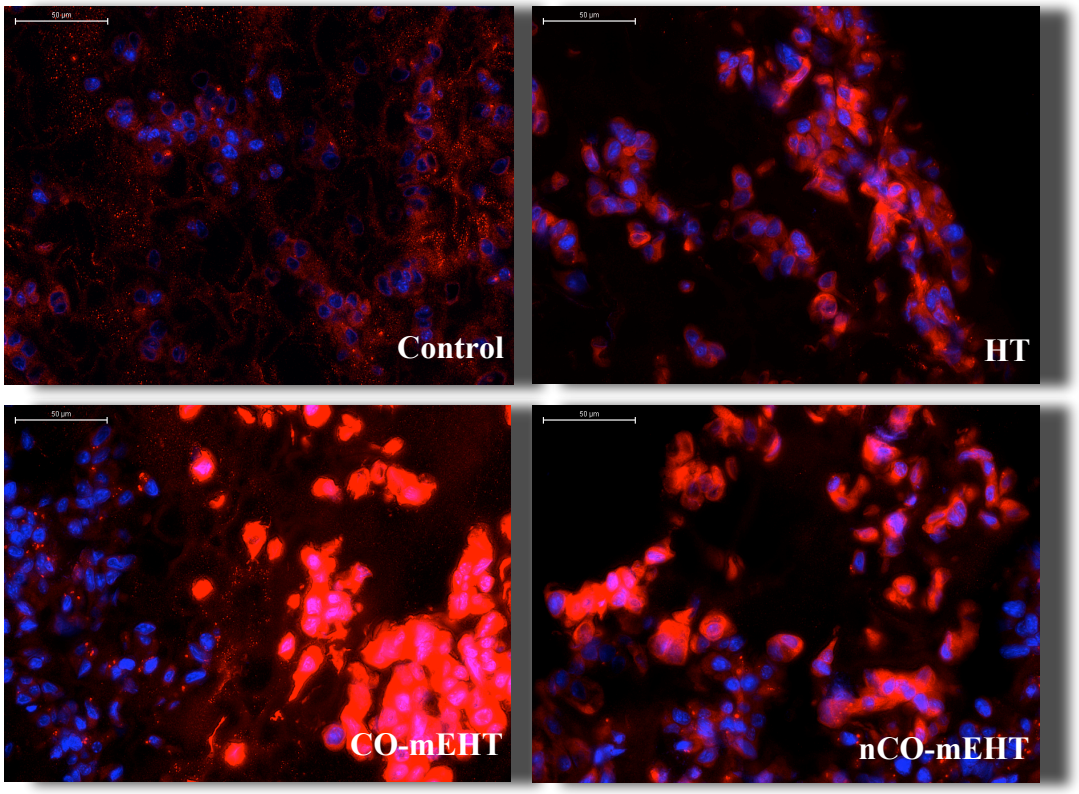

Figure 5.: These images show the results obtained from Hsp70 staining. Hsp 70 shows evident individuality between the samples. While an increased activation is seen in hyperthermia treated samples, m-EHT cells show evident activation of the protein. While nCO-mEHT shows higher levels of staining it is dispersed compared to CO-mEHT showing selected areas of specificity in its effect. Magnification is indicated in the scale bar in each picture.

6.3.3. BAX protein staining

BAX protein localisation in the control samples was seen predominantly in the mitochondria. In very rare occasions in some cells the protein concentrated into small particles in the cytoplasm. In the HT samples the particle formation of the protein has been seen in higher density than in the control cases. However the distribution of these cells was equal in the CO-mEHT samples (Figure 6.).

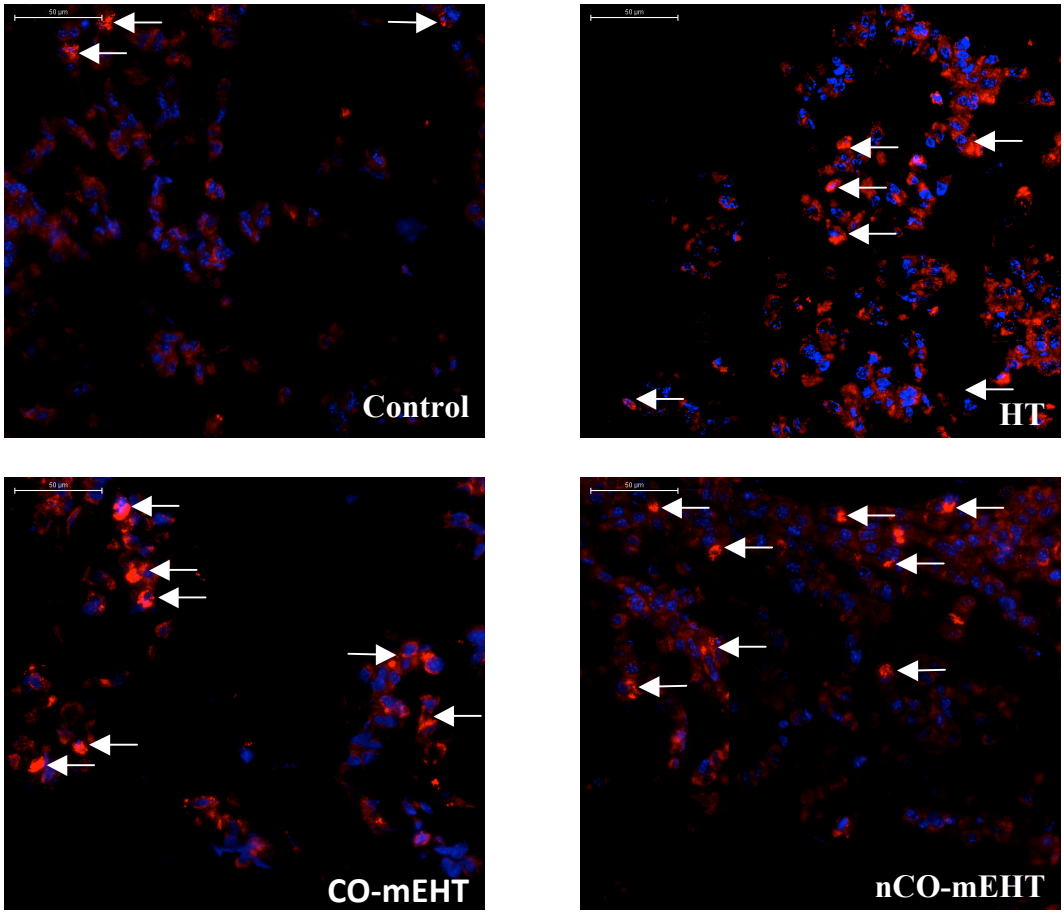

Figure 6.: The images show the result of BAX staining of the samples. Arrows are indicating the cells or cell groups, where the protein formed granular configuration around the nuclei, hence the dissolved BAX in the cytosol concentrating on the surface of the mitochondria. Magnification is indicated in the scale bar in each picture.

6.3.4. TUNEL assay

TUNEL positivity was emerged in all the treated samples in comparison to the control samples. In case of HT slides, the distribution of the positive cells was constant throughout the sample. As for the CO-mEHT slides, positive reactions have been seen in localized groups of cells. In other areas, the density of the TUNEL positive cells did not differ significantly from the distribution found in the control samples (Figure 7.).

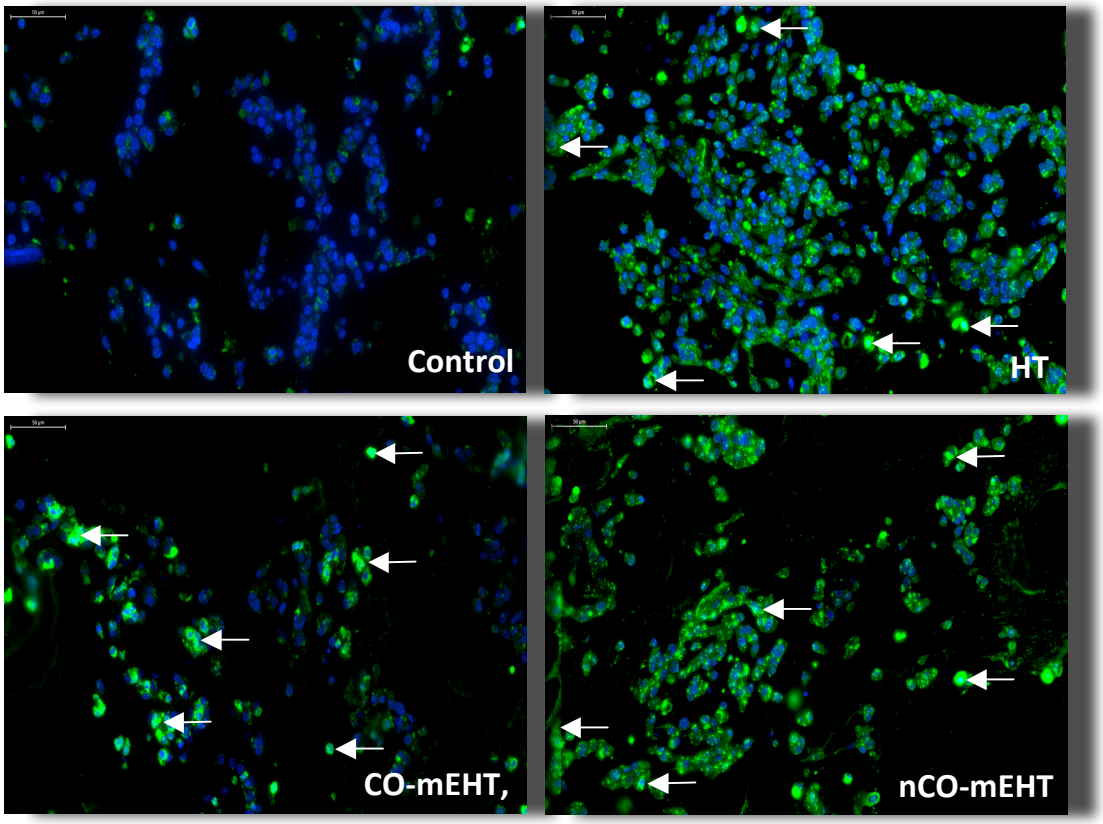

Figure 7.: The images show the results of the TUNEL assay. Arrows indicate the cells or cell groups that nuclei (blue) are co-localised with the TUNEL reaction (green), hence there is high amount of free ends of nucleic acid presented. This phenomenon in the nucleus is considered as a sign of the nucleic acid degradation caused by apoptosis. Magnification is indicated in the scale bar in each picture.

6.3.5. HMGB1 protein staining

During the evaluating of the results of the HMGB1 staining, we experienced a strong background reaction with the Matrigel on the slides. We considered this finding as an artifact. On the other hand, the real HMGB1 protein localization in the samples showed both nuclear and in some cases perinuclear/cytoplasmic localisation as well. In case of the treated samples, the cytoplasmic or perinuclear localization was seen slightly more often than in the control samples, although the difference was not significant. If we compare the treated slides with each other, the density of cytoplasmic localized HMGB1 signal showed up equally in the slides, however, in the CO-mEHT sample, the distribution of these signal was not even, it was concentrated to some parts of the slides, whereas in other regions the signal was weak (Figure 8.).

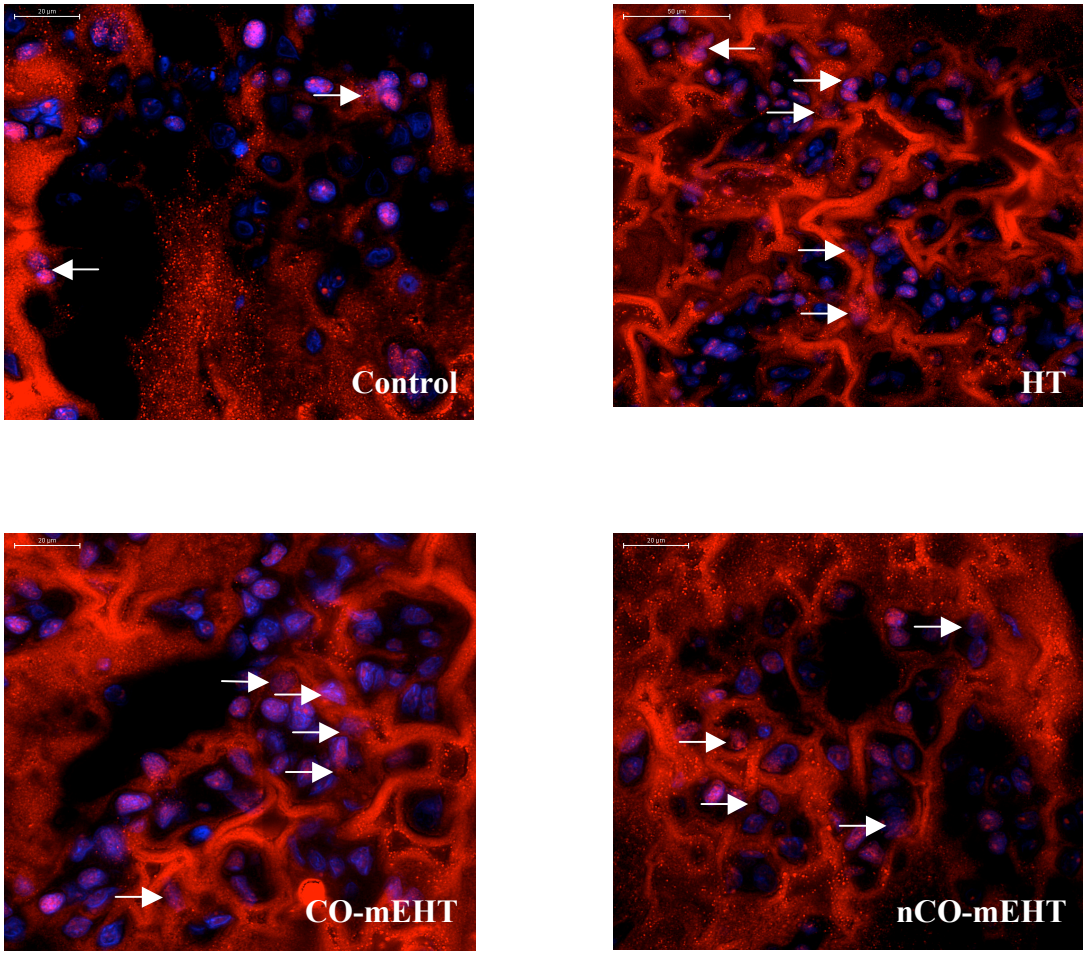

Figure 8.: The images are showing the result of the HMGB1 staining. Arrows are indicating the cells or cell groups where the protein is located in the cytoplasm. Magnification is indicated in the scale bar in each picture

7. Discussion

In our research we had multiple goals to complete. First, we wanted to create a viable 3D cell culture system that is capable of, at least partly, replicating living animal models in experiments. We also investigated the effects of the classic hyperthermia and mEHT treatment in alliance with this 3D cell system. Thirdly, we wanted to compare these results with the results obtained from earlier performed *in-vivo* studies.

According to our results, the 3D cell culture system proved to be viable and was able to survive for several days prior to the experiments. When inspected under a microscope the cell morphology showed similar structure to tumorous tissue in living *in-vivo*. Although in the culture only one kind of cell type was presented, the cell-nest structure with cellular adhesions between neighboring cell groups is typical of cancer cells. As the cells multiplication occurred, the cells invaded the gel layer nearly completely. It was seen however, that layering had become apparent in the sample. The more complex and developed cell structures were present in the upper regions of the gel layer where there was a higher amount of nutrients provided by the culturing medium. Here the metabolites were able to diffuse into the cells quicker due to the close proximity. In deeper regions, the complexity of the structure dropped as well as the size of the cell nests decreased. On the bottom of the culturing unit there were just some alone cells, cell pairs or triplets visible with minimal adhesions between these groups. This formation complies with the metabolic layering of a solid non vascularised tumor, where the superficial cell layers are able to develop and multiply whereas the inner layers have less nutrient access and more concentration of metabolites, therefore less viability of these cells is seen. In the centre of this kind of tumor usually there is a necrotic core, where the conditions are so poor for the inner cells that here the cells are unable to survive (Lin and Chang, 2008). In our system a part of this layering formation has been reproduced.

The experiments using direct heating hence classical hyperthermia and mEHT technique resulted differences in the data obtained. The activation of the Hsp 60 protein in all treated samples suggest that in all cases of treatment heat stress acted on the cells. Since there was no strong difference in Hsp 60 positivity between the HT and the mEHT treated cultures we can conclude that the cell's reaction is caused, at least partly, by the heat itself. The difference in the strength and distribution of Hsp 70 protein positive cells found in the HT, CO-mEHT and nCO-mEHT cells can also explained by the different heating method. The distribution of the Hsp 70 positive cells was even or nearly even in the HT and nCO-mEHT

samples. Here the heating happened evenly from an external heat source (HT) or the electro-magnetic energy reached the cells through a liquid layer, which provided the opportunity for the RF energy to spread more evenly (nCO-mEHT). Unfortunately due to technical reasons we were not able to conserve the exact topology of the original gel layer during the histology process. Despite this, in the case of CO-mEHT samples, there were extremely Hsp 70 positive region in the slides and also we found non-activated regions as well, sometimes right next to each other.

These results are in alliance with the physical fact, that in case of direct electric contact, the energy tries to move through the tissue by the shortest possible route. The cells positioned in the direct line of this route will suffer much higher energy density, hence more heating effect than other cells. Also slightly stronger Hsp 70 positivity was seen in the nCO-mEHT samples compared to the HT samples, so we can conclude that the mEHT treatment provides stress effect other than heat too for the cells. The presence of this extra effect also supported by the apoptotic signs proved by BAX and TUNEL reaction's results. As BAX protein translocation to the OMM is the sign of the degradation of the cell's energy system and the TUNEL signal over the nucleus is the sign of DNA fragmentation.

Both these findings in the same samples strongly suggest the activation of the apoptosis in the cells. In the treated samples we found both BAX and TUNEL positive cell in high amount compared to the control slides. It was also visible that the mEHT treated samples has more apoptotic-looking cells than the HT group samples. If we examine the distribution of these cells, again in the case of HT and nCO-mEHT samples, they are almost equal. In the CO-mEHT slides, we can see more localised reactions due to the more localised effect of the RF current. As for the HMGB1 relocation from the nucleus to the cytoplasm, in our examinations, we found weak signs in the slides, and there was not a significantly different effect between the treated and the controls samples. During the cell death process the relocation of the HMGB1 protein to the cytoplasm as well as the extracellular space. This helps the recognition of the dying cell by the immune cells and facilitates the antigen presentation process. It is possible, that in our experiment the 24 hours of incubation period was too short for the more pronounced translocation signal of HMGB1 to develop. This hypothesis also can be supported by the result of the HE staining, which revealed small amount of pyknotic or fragmented nuclei cell in the samples, but still in higher amount in the treated slides compared to the control.

If we compare the results gathered from our current investigation with data obtained from earlier C26 tumor mEHT-treatment animal experiment, we can see high level of similarity between the results (Table 2.).

Table 2.: Comparison of results gathered from the current experiment and earlier examination (Kővágó et al., 2014). (* difference between CO-mEHT and nCO-mEHT results respectively)

Examined protein/staining	***In-vitro* 3D culture**	***In-vivo* mouse model**
HE	+	+++
Hsp 70	+++/++*	+++
HMGB1	+/-	++
TUNEL assay	++	+++

In the living animal model all the signs suggesting strong effects due to external stress, most probably caused by the mEHT treatment. In both experiments the Hsp70 levels increased to high levels and TUNEL positive cells and areas were present in the samples. However, the data show differences in the number of pyknotic cell seen after HE staining and in the levels of trans-located HMGB1 protein amount.

Considering just the mentioned results it is uneasy to explain these differences. Most probably one cause of this is the fact, that the *in-vitro* model consisted only C26 tumor cells, whereas the tumor in the living animal besides of the artificially inoculated C26 cells also contained healthy cells from the mouse and active immune cells. The complex effect of the mEHT treatment was able to act on more types of cells in the *in-vivo* model, thus the cause of the previous findings can be not just the effect of the modulated RF current to the tumor cells, but also the effect of it to the healthy cells and the cell-cell interactions between the tumorous and healthy cells. This does not include the possible influencing capabilities of the real life tumor structure and blood circulation in the area. This comparison underlines that although the 3D cell culture technique is far more similar to the *in-vivo* circumstances than the mono-layer cell cultures, there are still structural and functional differences between multilayer cell cultures and living tissues or organs.

To summarize the conclusions of our experiment, we were able to successfully create 3D cultures which were and viable to be used as a test subject in mEHT experiments. When the treatments were done we see clear evidence that the cultures are capable of reenacting as *in-vitro* tissue. The results of the stainings demonstrated the expected conclusion of the treatment. There is there clear evidence the cells have undergone a stress and have degraded

either by Hsp70 induced apoptosis or nuclear degradation. Both of these mechanisms lead us to conclude that the modulated electro hyperthermia has strong potential for oncological treatment. We demonstrated that gel scaffold 3D cell cultures, despite of certain limitations are capable to simulate the properties of the *in-vivo* tissue and is has strong possibilities to substitute some animal experiments in the future. For this to happen further investigations and refinements of the experimental 3D cell culture systems should be conducted.

8. Summary

Multilayer or 3D cell culture systems have been used in research and biotechnology for several years, replicating solid tissues much better than a monolayer cell-cultures.

Modulated electro-hyperthermia (mEHT) is a special treatment method of tumors, which utilises electromagnetic energy to interfere with neoplastic tissues. The source of heating is impedance coupled electromagnetic energy. The tumor selectivity of mEHT is due to increased metabolites resulting in a higher electric conductivity than in normal tissues.

Our goal was to compare the effects of mEHT on 3D cell culture to the results gained from *in-vivo* experiments. We used Matrigel (a protein extract of Engelbreth–Holm–Swarm tumor), as an extracellular matrix scaffold for maintaining a colon carcinoma cell line, C26. We formed four treatment groups: HT group, heat treated by conventional hyperthermia; Non-cont. mEHT group, mEHT treated using non-contact electrodes; Cont. mEHT group, mEHT treated using contact electrodes and a C group of untreated cells kept outside the incubator at 37oC. All groups consisted of 3 samples.

The cultures were micrographed before treatment and 24h after treatment. Samples were fixed in formalin and embedded into paraffin blocks. Hematoxilin-eosin (HE) stained cell block sections for examination for basic cell morphology, cell connections and counting the proportins of living and dead cell. Immunohistochemistry (IHC) stainings were performed to identify the amount and morphology of the proteins Hsp60, Hsp70 and BAX. TUNEL assay was also used to identify the apoptotic cells using DAPI to mark the cell nuclei.

In cultures examined right before formalin fixation, typical cellular structure of the tumor were seen. The examination of the HE stained slides showed no significant change in the cell morphology compared to the control samples, except that in the treated slides there were more pyknotic nuclei or fragmented apoptotic bodies. As for IHC stainings, Hsp70 positivity emerged in the treated samples, especially in case of cont. mEHT group. TUNEL assay showed that apoptotic nuclei can be found in high numbers in case of treated goups, and BAX also identified apoptotic signs in the treated samples.

Our results showed high similarity to previous *in-vivo* experiments, thus we can conclude that our 3D cell culture model reflects living animal models.

9. Összefoglalás

A 3D sejttenyészetek szerkezetüknél fogva sokkal jobban utánozzák a magasabbrendű szervezet viszonyait, mint az egyrétegü szövetkultúrák. Emiatt régóta alkalmazzák ezeket kutatási és biotechnológiai célokra. Létrehozásuknak egyik módja a mesterséges extracelluláris mátrix-gél használata, erre a célra a legelterjedtebb anyag az Engelberth-Holm-Swarm tumorból kivont fehérjekomplex, mely Matrigél-ként is ismert.
A modulált elektro-hiperthermia (mEHT) tumorellenes kezelési eljárás, mely modulált elektromágneses energiával módosítja a daganatsejtek müködését. A célszövet a kezelés során felmelegszik a közölt impedancia-csatolt elektromágneses energia következtében. A hőhatáson kívül az eljárás a daganatos sejtek jelátviteli útjainak befolyásolásával éri el a kívánt hatást.

Vizsgálatunkban össze kívántuk hasonlítani az mEHT 3D sejttenyészetre kifejtett hatását korábbi, *in-vivo* kísérlet eredményeivel. A kísérletben Matrigél alapú 3D szövetkultúrát hoztunk létre egér colon-adenocarcinoma eredetű C26-os sejtekből és négy kísérleti csoportot alakítottuk ki: HT; Non-cont mEHT; Cont-mEHT; és egy kontroll csoport, (C), melyet 37^{o}C hőmérsékleten a kezelések időtartamának megfelelően. Minden csoportban 3 minta szerepelt.

A mintákról a kezelések elött és 24 órával később natív mikroszkópiás felvételeket készítettünk, majd szövettani feldolgozás keretében hematoxilin-eozin (HE) festést végeztünk a sejtek morfológiájának, kapcsolatainak felderítése és az élő és elhalt sejtek számának meghatározására. Immunhisztokémiai (IHC) festéseket végeztünk HSP60, HSP70 és BAX fehérjék mennyiségének, lokalizációjának megismerésére illetve Tunel vizsgálatot hajtottunk végre az apoptotikus sejtek kimutatására, a sejtmagokat DAPI-val jelöltük.

A kultúrák natív képein a daganatsejtekre jellenző, fészkes sejtcsoportokat látunk, melyek egymással sejtes hidakkal kapcsolódtak és a tér minden irányába kiterjedtek. A HE metszetek vizsgálatakor a kezelt mintákban több piknotikus magot illetve apoptotikus testet lehetett látni. Az IHC vizsgálatok eredményeként a HSP70 fehérje felülregulációját figyeltük meg a kezelt, különösen a "Cont-mEHT" mintákban, ahol helyenként nagyon erős jelet kaptunk. A Tunel assay apoptózisra utaló eredmény hozott a kezelt mintákban és a BAX festés is megerősítette a nagyszámú apoptotikus sejt jelenlétét.

Eredményeink nagyfokú hasonlóságot mutatnak a korábbi, élő állatokon végzett hasonló kezelések után tapasztaltakkal, így levonhatjuk a következtetést, hogy a 3D sejtkultúra bizoyos körülmények között alkalmas az *in-vivo* módszerek kiváltására. Valószínűleg azonban ezek a módszerek sem tudják teljesen kiváltani az állatkísérleteket a jövőben.

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