Universidade do Porto
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EVALUATION OF HFT'S *IN VITRO* AND EFFECTS ON THE CELLS VIABILITY

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Those diseases that medicines do not cure are cure by knife

and those that the knife cannot cure are cure by fire.

And those that fire does not cure are to be reckoned wholly incurable

Adams, F. *The Genuine works of Hippocrates* (1886)

To my Parents and Sister

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Resumo

A termoterapia altamente focalizada (HFT) é uma terapia que utiliza a indução exógena de calor na eliminação do tecido tumoral. Estudos in vitro descrevem efeitos globais, desde a estrutura à função das células submetidas à hipertermia. Estes efeitos dependem da linha celular, temperatura, tempo de exposição e tempos escolhidos para estudar a reacção de choque térmico.

O objectivo deste trabalho foi o estudo do efeito da hipertermia no comportamento de duas linhas celulares, células de melanoma B16-F10 e células de tumor mamário 4T1. Também se pretendeu estudar o efeito de substâncias tóxicas libertadas para o meio após o tratamento de hipertermia.

As duas linhas celulares, B16-F10 e 4T1, foram submetidas a um tratamento de hipertermia a 45ºC, por 30 minutos, no dia 5 de cultura. Ambas, culturas controlo e culturas sujeitas a aquecimento, foram mantidas a 37ºC por 14 dias. O comportamento celular foi avaliado pelo estudo da viabilidade/proliferação, número de células aderentes, mecanismo de morte celular e morfologia. O meio condicionado de culturas sujeitas a aquecimento foi administrado a novas culturas, e as alterações na viabilidade foram estudadas nas duas linhas celulares.

Os resultados demonstraram que a reacção de choque térmico, a 45ºC, é observada imediatamente após o tratamento. Observou-se um decréscimo na viabilidade/proliferação chegando a valores 60% e 46% após 24 horas, para as linhas celulares B16-F10 e 4T1, respectivamente. Estes efeitos foram acompanhados por alterações no tipo de morte celular e na morfologia. As células iniciaram o processo de morte por apoptose secundária imediatamente após o tratamento, apresentando valores de 77% e 33% às 24 horas, para as células B16-F10 e 4T1, respectivamente. As células que não foram destruídas durante as primeiras 24 horas apresentaram um processo de morte celular por apoptose primária após alguns dias. O meio condicionado, recolhido imediatamente após o tratamento de hipertermia (0 horas) nas culturas de células B16-F10 e o meio condicionado recolhido após 0, 3, 6 e 48

horas nas culturas de células 4T1, também induziram o decréscimo da viabilidade/proliferação de células da mesma linhagem.

Em conclusão, o tratamento de hipertermia a 45ºC origina uma reacção de choque térmico que leva à destruição celular por duas vias diferentes. Primeiro induz danos celulares levando à morte celular por apoptose e alguma necrose. A morte celular inicia um segundo efeito de danos celulares devido à libertação de substâncias tóxicas para o meio de cultura.

Abstract

High focalized thermotherapy (HFT) is a new therapy to cancer treatment. It uses exogenous heat induction as therapeutic agent against tumour tissue. In vitro studies have described that cells submitted to hyperthermia show global effects in their structure and function. These effects are dependent of cell line, temperature, time of exposure and also the time-points chosen to assess heat shock reaction.

The aim of this work was the study of the effects of hyperthermia treatment in cell behaviour of two cancer cell lines, B16-F10 melanoma cells and 4T1 mammary tumour cells. The toxic effects of substances that leaked to the medium after hyperthermia treatment was also studied.

B16-F10 melanoma and 4T1 mammary tumour cell lines were submitted to a hyperthermia treatment at 45ºC for 30 minutes. Control cultures and heat-treated cultures were kept at 37ºC up to 14 days. Cell behaviour was assessed by studying cell viability/proliferation, number of adherent cells, mechanism of cellular death and morphology. Conditioned media from cultures submitted to hyperthermia were administered to new cultures, and alterations in viability/proliferation were studied for both cell lines.

Results demonstrated that the heat shock reaction at 45ºC is observed immediately after treatment. Cells showed a decrease of viability/proliferation of 60% and 46% in B16-F10 and 4T1 cell line, respectively, after 24 hours. These effects were followed by alterations in mechanism of cellular death and morphology. Cells began a process of cellular death by secondary apoptosis immediately after treatment, with values of 77% and 33% at 24 hours, for B16-F10 and 4T1 cells lines respectively. The remaining cells showed a process of cellular death by primary apoptosis after few days. Also, conditioned medium from cultures submitted to hyperthermia have shown to affect cellular growth in the two cell lines. B16-F10 conditioned medium collected 0 hours after treatment and 4T1 conditioned medium collected 0, 3, 6 and 48 hours after treatment induced a decrease in cellular viability/proliferation.

In conclusion, hyperthermia treatment at 45ºC set a heat shock reaction that leads to destruction of the cells by two different ways. First, it induce damage in cells leading to a process of cellular death by apoptosis and some necrosis; this will lead a second process of cellular damage induced by toxic substances that have leaked to the medium.

Content

Abbreviations

Abs…………Absorbance

BSA…………Bovine serum albumine

CLSM…………Confocal laser scanning microscopy

DMSO…………Dimethylsulphoxide

DNA…………Deoxyribonucleic acid

EDTA…………Ethylenediamine tetraacetic acid

FBS…………Fetal bovine serum

HFT …………High focalized thermotherapy / Termoterapia altamente focalizada

HSP…………Heat shock protein

HSP70…………Heat shock protein 70

HUVECs…………Human umbilical vein endothelial cells

LDH…………Lactate dehydrogenase

MRE11…………Double-strand break repair protein

MTT…………3-(4,5-Dimethylthiazol-2-yl)-2,5,diphenyltetrazolium

PBS…………Phosphate buffered saline

PI…………Propidium iodide

RNase…………Ribonuclease

ROS…………Reactive oxygen species

α-MEM…………α – minimal essential medium

1 Introduction

1.1 *Hyperthermia therapy*

Cancer is one of the major causes of mortally in the industrialized countries, although, there have been advances in cancer therapeutics, surveillance and prognosis, so it's clearly necessary to improve all these areas.

Currently, there are a variety of methods for cancer therapy as surgery, radiotherapy, chemotherapy, immunotherapy, gene therapy, hyperthermia, phototherapy, etc (Jain, 2001; Liu *et al*., 2005). Chemotherapy and radiotherapy are the two modalities most commonly used in cancer treatment. Although their proven effectiveness in tumours detected early, with tumours detected in a late phase these two therapies aren't very effective and, also, they have a variety of side effects (Le *et al.,* 2004; Omand and Meredith, 1994; Sitzia *et al*., 1995). So there is a necessity to start researching new cancer therapies, which could increase the success in treatment of advanced cancer and with lower side effects.

One of these new therapies is hyperthermia which uses exogenous heat induction as therapeutic agent (Vertrees *et al*., 2007). It can be used alone or as adjuvant to other therapies (especially chemotherapy and radiotherapy) in the treatment of cancer (Hildebrandt *et al*., 2002). Hyperthermia can be administered with local, interstitial, whole-body and perfusion techniques, and they differ in the way heat is applied to the patient. All this techniques are still less efficient compared to other therapeutic procedures already established like radiotherapy and chemotherapy and for this reason hyperthermia has been mostly used as adjuvant to the last ones (Hildebrandt *et al*., 2002; Van der Zee, 2002).

Localized techniques apply heat only in the tumour using electromagnetic energy or ultrasounds (Van der Zee, 2002). High focalized thermotherapy (HFT), a localized technique, allows heating the tumour without affecting the surrounding tissue. In this therapy, a ferromagnetic material is applied to the tumoural tissue, generating heat when an alternating magnetic field is applied (Almeida *et al.,* 2002; Cavalheiro *et al.*, 1996).

The use of hyperthermia is controversial and presents significant technical challenges. Scientific reports present contradictory results relatively to hyperthermia therapeutic potential, some attesting its usage and some showing no therapeutic effect. This is probably due to the lack of knowledge correlating the hyperthermia effects at cellular level and those at tissue level, which are more complex. Another problem with this therapy is the determination of the "therapeutic thermal window", a combination of temperature and time of exposure to heat treatment that would destroy the target tissue but not the normal tissue surrounding it (Vertress *et al*., 2007).

1.2 *Therapeutic* thermal window

In humans, there are three important temperature ranges, and they will help defining the "therapeutic thermal window". Healthy humans have a temperature approximately of 37 ºC, and when diseased the temperature can go up to 41.8 ºC. This elevated body temperature defined as fever, inferior to 41.8 ºC, is a basic physiologic function that has a beneficial role enhancing the body defence (Blatteis, 2002; Mackwiak, P. A., 1994). Temperatures above 44 -45 ºC have been shown to lead to protein denaturation, culminating in cell death, and are consider being deleterious to the body. Above this temperature, both normal and neoplastic tissue will be destroyed. So, the "therapeutic thermal window" would be located between 41.8 ºC and 44 ºC, between beneficial fever and deleterious hyperthermia (Figure 1). At this temperature range hyperthermia therapy may exert a beneficial role, defined as destruction of the target tissue with minimal to no effect to the normal tissue (Vertress *et al*., 2007).

Also, the therapeutic thermal window will be defined by time of exposure to heat treatment. Even at lower temperatures, extensive exposure to heat treatment will be deleterious to cells and tissues (Rong and Mack, 2000; Vorotnikova *et al.,* 2006; Yonezawa *et al.*, 1996; Rong and Mack, 2000)

This therapeutic thermal window still leads to a lot of controversy, as different reports show different effects. The reasons for these problems should be the use of different techniques to administer the heat, time and temperature of exposure, cell line, and method used to measure effects of heat treatment. Although this controversy, there has been a growing interest in this therapeutic and in the past few years there have been a lot of publications concerning the use of hyperthermia, from clinical trials to effect on culture cells.

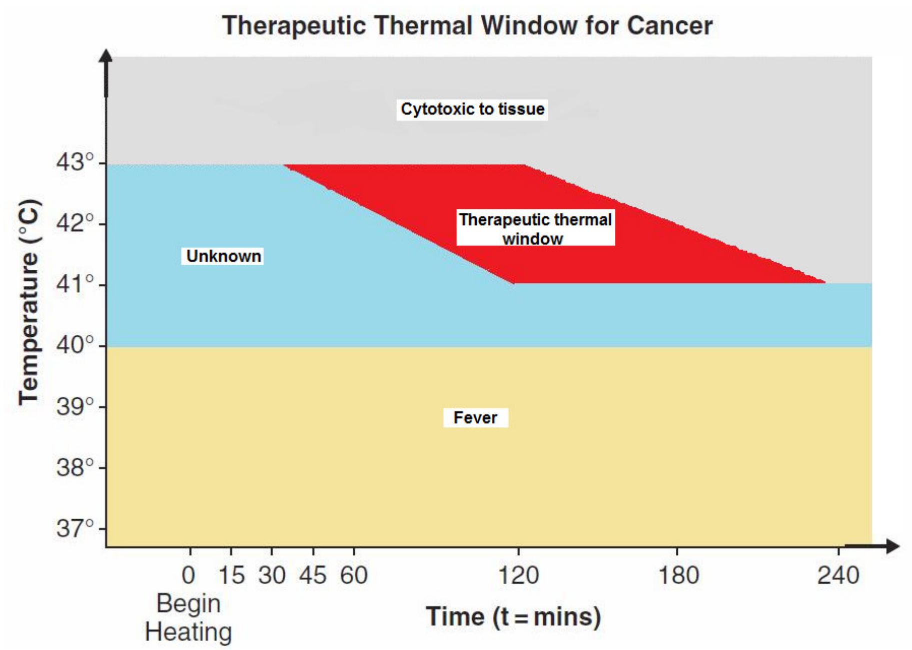

Figure 1. Temperature ranges with physiologic relevance. Therapeutic thermal window (red) will, possibly, be defined between 41ºC and 43ºC, limited by exposure time (Vertress *et al*., 2007).

1.3 In vivo studies

In vivo studies have been made in a variety of tumour models as melanoma, glioma, carcinoma, osteosarcoma, prostate cancer, mammary cancer and hepatoma, most of them showing some benefit in the use of hyperthermia (Hamaguchi *et al.,* 2003; Ito *et al*., 2003a; Kawai *et al.*, 2005; Matsuoka *et al.*, 2004; Motoyama *et al*., 2008; Saito *et al.*, 2008; Shinkai *et al.,* 1999; Yanase *et al.,* 1998a).

There have been several reports concerning the effect of hyperthermia in the treatment of melanoma. In a previous work, mice bearing melanoma where treated with hyperthermia at 43ºC for 30 minutes (Ito *at el*., 2003a). Ito and co-workers (2003a) described that this temperature wasn't enough to increase the survival rate of the mice with this type of tumour. Although the results weren't has god as expected they found that there is an augmentation of HSP70 (heat shock protein 70), which is involved in immunological reaction against the tumour (Ito *et al*., 2003a; Ito *et al*., 2003b; Ito *et al.*, 2004). Saito *et al.* (2008) found that, although one treatment (43ºC, for 30 minutes) wasn't enough to treat the tumour, using this therapy twice a week for 4 weeks suppresses tumour growth and survival rate increases. A higher temperature, of 46ºC, applied once a day for two days originated a complete regression of the tumour in 90% of the mice (Ito *et al*., 2003b; Suzuki *et al*., 2003).

Rats with mammary cancer when submitted to hyperthermia treatment at 43ºC for 30 minutes (three times) exhibited decrease in tumour growth. In a few cases was observed a regrowth of tumours but they regressed after some weeks indicating an induced immunological antitumour activity mediated by the treatment (Motoyama *et al*., 2008). A temperature of 45ºC for 30 minutes has proved to have a better outcome to the treatment of mammary carcinoma, as described by Ito *et al.* (2003c). Mice treated with this therapy would achieve a completed regression of tumour and also acquired antitumour immunity (; Ito *et al.*, 2006; Manjili *et al.*, 2002).

Another well-known tumour model, the glioma, was also used to test hyperthermia effect. Using a temperature of 43ºC for 30 minutes, Yanase and co-workers (1998a) have shown a regression between 20% and 87% dependent of treatment received (one and three times, respectively). They also described an immunological effect, when rats bearing glioma in each femur were treated at the same temperature. In this case one of the tumours was treated and, although the other tumour stayed at 37ºC during hyperthermia treatment, the tumour also regressed (Figure 2) (Yanase *et al.,* 1998b). This effect has been attributed to a response of the immune system (Yanase *et al.,* 1998b; Shinkai *et al.,* 1999).

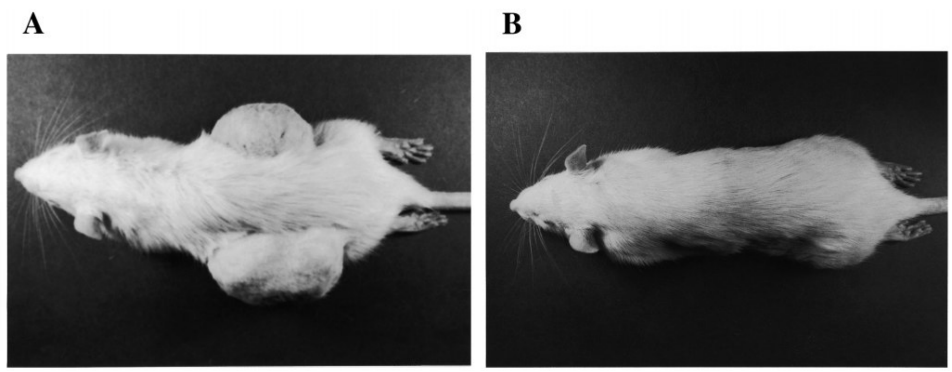

Figure 2. Tumour growth in rats bearing glioma, 28 days after inoculation with magnetic particles. A) Rat from control group, inoculated with magnetic particles in one of the tumours. B) Rat inoculated with magnetic particles in one of the tumours and submitted to hyperthermia treatment at 43ºC for 30 minutes (Yanase et al., 1998b).

In vivo studies have shown that in hyperthermia treatment at lower temperatures, as 43ºC, the regression of tumour can only be observed if the treatment is applied more than one time and higher temperatures can lead to tumour regress with only one treatment. Both temperatures used in the works described above didn't affect the normal tissue surrounding the tumour, leading to a possible benefit of using higher temperatures to treat the tumour with less applications of the treatment. Also, hyperthermia treatment leads to a second deleterious effect to the tumour; it can originate an immune reaction leading to an effect similar to a specific anti-tumour vaccination.

1.4 In vitro studies

In vitro studies have also shown the effects of hyperthermia in the tumour and normal cells. In the cells, heat shock effect has been found to be global. The effects involve almost all known structural and functional systems in cells, from activation or inhibition of certain gene expression, protein degradation and denaturation, alterations in cellular ion homeostasis and culminating in cellular death when the majority of cell system fails (Figure 3) (Kanamori *et al.*, 2003; Nakayama *et al.,* 1996; Milani *et al.*, 2005; Rong and Mack, 2000; Vorotnikova *et al.*, 2006; Zhou *et al.,* 2007). Hyperthermia effect is complex and alterations in one compartment of the cell can lead to other effects.

1.4.1 Cell membrane and cytoskeleton

Hyperthermia was found to affect fluidity and stability of cellular membranes and inhibit the function of transmembranal transport proteins and cell surface receptors (Hildebrandt *et al.,* 2002). The alterations in the membrane will affect the transmembrane transport of ions (as Na^+/H^+ transport) and intracellular pH, as found by Babsky *et al.* (2005). Hyperthermia treatment at 45ºC for 30 minutes increased, irreversibly, the concentration of Na^+ in the interior of the cell and decreased the pH of RIF-1 cells, indications of possible alterations in cellular membrane.

Alterations in cytoplasmic and mithocondrial membrane will lead to the generation and release of ROS (Reactive oxygen species) and increase of intracellular concentration of Ca^{2+} (Figure 3) (Grasso *et al.,* 2003). ROS are highly reactive substances that can interact with cellular molecules like proteins, leading to protein destabilization (Halliwell *et al.,* 1999; Thannickal and Fanburgo*,* 2000). When these protein alterations occur in the nucleus, especially in proteins involved in DNA transcription, replication and repair, they will result in a increase of unrepaired DNA damage contributing to chromosome aberrations, genomic instability and cell death (Roti Roti, 2008).

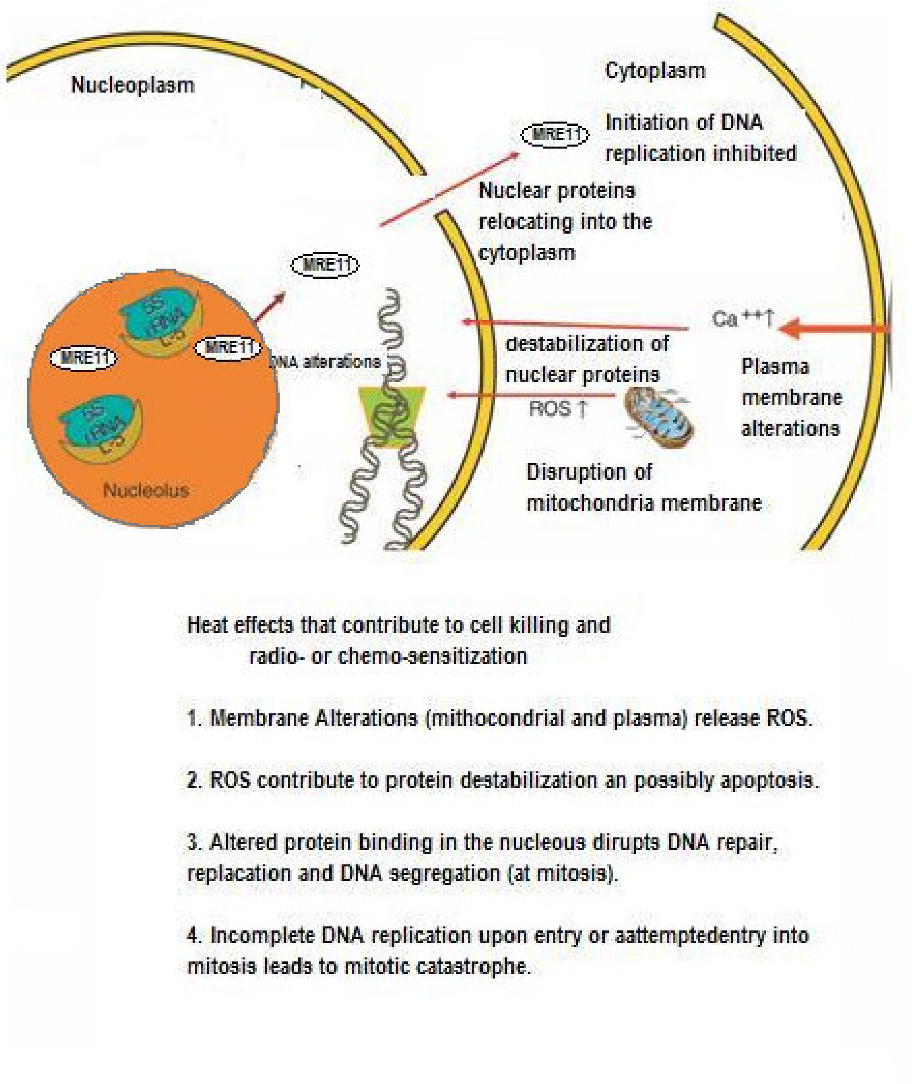

Figure 3. Examples of the hyperthermia effects that contribute to cell killing and sensitization of cells to radiotherapy and chemotherapy (Adapted from Roti Roti, 2008)**.**

Heat treatment revealed to induce various changes in cytoskeletal organization (cell shape, mitotic apparatus, intracytoplasmatic membranes such as endoplasmatic reticulum and lysosomes) (Hildebrandt et al., 2002). Hyperthermia treatment of thyroid carcinoma cells at 43ºC induced depolymerazition of actin filaments, intermediate filaments and microtubules, demonstrating that this treatment induces disruption of integrin-mediated actin cytoskeleton assembly (Huang et al., 1999).

1.4.2 Cellular proteins and nucleic acids

Several studies have reported that hyperthermia can affect proteins. It can affect unfolding and exposing hydrophobic groups of proteins and aggregation of unfolded proteins due to the interactions of the hydrophobic groups (Roti Roti, 2008). This aggregation of proteins can include proteins not directly altered by hyperthermia, thereby affecting a larger number of proteins than those directly altered by heat shock. Submission of cells at temperatures between 40 – 47ºC causes proteins to be unfolded, exposing hydrophobic groups, which can then interact forming aggregates. These heat effects on proteins are, in principle, reversible by the presence of molecular chaperones, such as heat shock protein 70 (HSP70) (Kampinga *et al.,* 1994; Roti Roti, 2008).

Also, hyperthermia between 40 and 45ºC can cause delocalization of a DNA repair protein MRE11 (Figure 3) from the nucleus into the cytoplasm (Roti Roti, 2008; Xu *et al.,* 2007).

1.4.3 Cell viability/proliferation

As described above, hyperthermia originate alterations at all cellular levels and these alterations can be reversible or lethal. Hyperthermia treatment at different temperatures for different times alternate from cells that resists damage and can recover (low temperature and short exposure time) to cells that cannot compensate and undergoes predominantly apoptosis (higher temperature and short exposure time or low temperature and long exposure time) to cells that immediately undergoes necrosis (high temperatures and long exposure time) (Vorotnikova *et al*., 2006).

Hyperthermia can also affect viability and proliferation of culture cells, normal and tumour cells.

Human umbilical vein endothelial cells (HUVECs) submitted to hyperthermia at 43ºC for 120 minutes have no alteration in viability comparing to cells maintained at 37ºC. However, when submitted at temperature of 45ºC the viability was reduced by 50% after 120 minutes (Fukao *et al*., 2000). In the same report, Bowes cells (human melanoma cell line) showed to be more sensitive to hyperthermia, even at 43ºC, viability was reduced to levels of 70% comparing to control.

Sharif-Khatibi *et al.* (2007) described inhibition in growth of human erythroleukaemia cells (K562 cell lines) submitted to 43ºC (for 0, 30, 60 and 90 minutes) in a dose and time dependent manner, without affecting their viability. Treatment at 45ºC (for 0, 30, 60 and 90 minutes) has reduced both growth and viability, depending of time of exposure (dose) and time after hyperthermia treatment (Sharif-Khatibi *et al.,* 2007).

1.4.4 Mechanism of cellular death

Several studies have dedicated to the study of cellular death after hyperthermia, describing two different ways of death, apoptosis and necrosis, depending of temperature, time of exposure and cell line.

Cellular death occurs by two different ways, necrosis or apoptosis (Creagh *et al.,* 2000; Edinger and Thompson, 2004). Necrosis is marked by a passive pathological cell damage followed by an inflammatory response originated from the surrounding tissue. During the event of necrosis, necrotic cells lose the ionic control, swell and burst, releasing intracellular chemotactic substances, as proteolytic enzymes, and cellular debris into the surrounding tissue, evoking an inflammatory response that can lead to substantial killing of adjacent cells (Fink and Cookson, 2005; Majno and Joris, 1995). Differently, apoptosis is a genetically controlled, active death program. This process can be activated by cell damage or physiologically, contributing to maintain tissue haemostasis as well as preventing severe cell damage that could lead to cancer. Apoptotic cells don't lyses immediately; instead express on their intact membranes marker proteins that can target these cells for phagocytosis and rapid removal via on-site inflammatory cells and neighbouring cells (Wu *et al.,* 2006; Maderna and Godson, 2005). Cells undergoing apoptosis shrink, show nuclear condensation, and fragment into apoptotic bodies that are recognized and phagocytosed by other cells (Creagh *et al.,* 2000; Fink and Cookson, 2005; Majno and Joris, 1995).

Hyperthermia treatment at 43ºC (for 0, 30, 60 and 90 minutes) doesn't affect cellular death by apoptosis and necrosis of human erythroleukaemia cells (K562) (Sharif-Khatibi *et al*., 2007). This could be a result of the augmentation of HSP70 (heat shock protein) between 4 and 8 hours after heat treatment, observed by the authors. HSP70 can regulate growth, differentiation and cell death, and inside the cell it can also protect cell from stress as heat (Manjili *et* al., 2002; Schmitt *et al.*, 2007). Treatment at 45ºC increases cellular death by apoptosis and partially necrosis of erythroleukaemia cells. At this temperature there is no augmentation of HSP70 in the cell, so cells couldn't be protected from heat effects. Also, high levels of anti-apoptotic proteins, as Bcl-xl, where observed in this cell line, which would mediate the amount of cellular death, making this cells more resistant to hyperthermia cellular death (Sharif-Khatibi *et al.*, 2007; Strasser and Anderson, 1995).

Nijhuis *et al.* (2006) have described that the treatment at 43ºC during 1 hour leads human promyelocytic leukemic cells to have a fast heat-induced cellular death based on late apoptotic fraction, indicating permeabilization of the outer membrane. Also, they found an augmentation of pro-apoptotic Bax protein and decrease of Bcl-2 (anti-apoptotic protein).

Rong and Mack (2000) also describe the induction of apoptosis of Dunn osteosarcoma cell line after treatment at 43.5ºC for 1 hour, and apoptotic changes in cells started 6 hours after.

Differently from observation *in vivo*, *in vitro* studies shows a variety of results that are mostly dependent of cell line. Cell lines have differences in proteins involved in apoptotic process that can influence the results obtained. Also *in vivo* studies will be affected by the reaction of the immune system; this will also influence the result obtained. Possibly these factors are the reason in differences obtained from *in vivo* and *in vitro* studies.

From the reports described, hyperthermia at 43ºC has less effect on the cells and depends of the immune system to destroy the tumour. At 45ºC, cells are destructed by the heat and the heat shock reaction takes place immediately so the treatment of tumours *in vivo* was also faster. Although this is a higher temperature and less used in treatment, there were no reports saying that this temperature would affect normal tissue surrounding the heated tissue. So, in this work it was used a temperature of 45ºC because of the fastest outcome observed *in vivo* (compared to 43ºC treatment) and there weren't found side effects in using this temperature.

2 Objective

The objective of this work was to study hyperthermia effect in two cell lines, B16-F10 melanoma cells and 4T1 mammary tumour cells. Alterations after hyperthermia treatment, at 45ºC for 30 minutes, where determined by testing viability/proliferation, number of adherent cells, mechanism of cell death and morphology. This work had the objective of determining the period of the heat shock reaction in tumour cells and the progression of the culture after hyperthermia treatment. Further, by using conditioned medium from B16-F10 melanoma and 4T1 mammary tumour cell cultures submitted to hyperthermia, the toxic effect of substances that leaked to the medium from damaged cells was also accessed in the viability/proliferation of the same cell line.

3 Materials and methods

3.1 Materials

All cell culture chemicals and supplies were purchased from Merck and Sigma-Aldrich (St. Louis, MO). The In vitro toxicology assay kit lactate dehydrogenase based (Tox7) was purchased from Sigma-Aldrich (St. Louis, MO). The TACS™ Annexin V-FITC Apoptosis Detection Kit was purchased from R&D Systems (Abingdon, UK). All tissue culture flasks and plates were obtained from Corning (Corning, NY).

3.2 Cell Culture

Cell lines B16-F10 melanoma cells from C57BL/6J mice and 4T1 mammary tumour cells from BALB/cfC3H mice were purchased from ATCC. Cells were cultured in 90 mm culture plates and were maintained in standard culture conditions, i.e. α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS; Gibco/BRL), 2.5 μgmL^{-1} fungizone, penicillin-streptomycin (100 $IUmL^{-1}$ and 10 $mgmL^{-1}$, respectively), and incubated in a humidified atmosphere of 5% CO_2 in air at 37ºC. Culture medium was changed twice a week.

At 70 to 80% cell confluence, adherent cells were enzymatically released which consisted in the following steps: medium was removed and the cell layer was washed with phosphate buffered saline (PBS, at 37ºC), a solution of 0.04% trypsin in 0.25% EDTA (1 mL) was added to detach adherent cells, and the culture plates were incubated at 37ºC in a humidified atmosphere (5% CO_2 in air) for 5 minutes. Complete culture medium (2 mL) was added to the culture plates to stop the enzymatic reaction. The resulting cell suspension was cultured at a density of 5 x 10^3 $cellcm^{-2}$, and cell cultures were used to perform the hyperthermia studies.

3.3 Hyperthermia treatment

B16-F10 melanoma cells and 4T1 mammary tumour cells, seeded at 5 x 10^3 cellcm^{-2}, were cultured in the experimental conditions described above. At day 5, 50 – 60% confluence, cell cultures were submitted to a hyperthermia treatment. In this procedure, culture plates, sealed with parafilm sealing film, were totally submerged in a water bath and incubated at 45ºC (±0.1ºC) for 30 minutes. Heat-treated cultures were cultured for further 9 days at 37ºC, and the medium was changes twice a week. Control cultures, which were kept at 37ºC, were evaluated simultaneously. Figure 4 shows a diagram of the experimental protocol used in the hyperthermia experiments.

3.4 Effect of hyperthermia treatment in the cell behaviour

B16-F10 melanoma and 4T1 mammary tumour cell cultures, both control and those submitted to the hyperthermia treatment, were evaluated for cell morphology, cell viability/proliferation, number of adherent cells and mechanism of cell death. Cultures were tested throughout the culture time, before and after the hyperthermia treatment. Heat-treated cultures were evaluated immediately after the heat treatment and after 3 h, 6 h, 24 h, 48 h, 6 days and 9 days.

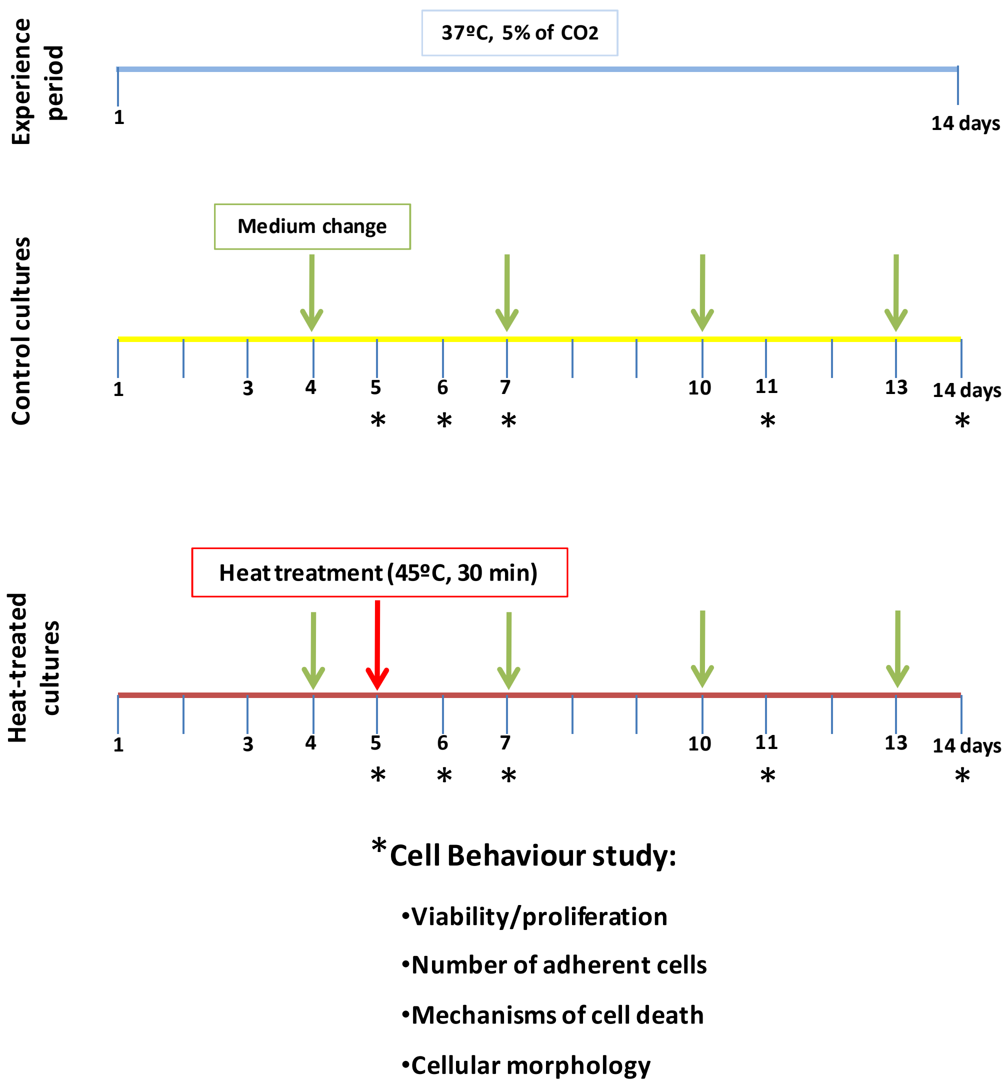

Figure 4. Diagram of the experimental protocol used in the hyperthermia experiments. → : medium change. → : hyperthermia treatment, at 45ºC for 30 minutes.

3.4.1 Cell viability/proliferation

B16-F10 melanoma cells and 4T1 mammary tumour cells were cultured (5 x 10^3 $cellcm^{-2}$) in 96-well culture plates in standard culture conditions. Cell viability/proliferation of control and heat-treated cultures was analyzed with MTT assay and LDH assay, at defined time points.

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5,diphenyltetrazolium) assay is a method to evaluate viability, proliferation and activation of cells. This method is based in the capability of mitochondrial dehydrogenase, in live cells, to transform the MTT in a dark blue formazan product (Morgan, 1997; Mosmann, 1983).

MTT (0.5 $mgmL^{-1}$) was added to each well, and the culture plates were incubated for 2 h at 37ºC. After the incubation period, the culture medium was removed; the formazan salts were dissolved with 100 µL of dimethylsulphoxide (DMSO) and the absorbance was determined at λ = 600 nm on a ELISA reader (Denley, model Wellscan WS 050). Results were expressed as Absorbance (Abs) and Percentage of inhibition of cell viability/proliferation in heat-treated cultures (%) which was calculated has follows:

% = (Absorbance in Control – Absorbance in Heat-treated cultures) x 100

The lactate dehydrogenase (LDH) assay is a mean of measuring either the number of cells via total cytoplasmatic lactate dehydrogenase or membrane integrity as a function of the amount of cytoplasmic LDH released into the medium (Legrand *et al.*, 1992). The assay is based on the reduction of NAD by the action of LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium dye.

Determination of the total LDH was performed in control and heated culture plates using the In vitro toxicology assay kit lactate dehydrogenase based (Sigma-Aldrich; St. Louis, MO). To determined the total LDH in the culture, LDH assay lysis solution (1/10 of the culture medium volume) was added to the wells and the plates were incubated at 37ºC for 45 minutes. Medium was transferred to eppendorfs and centrifuged at 250 x g for 4 minutes to pellet debris. For determining the LDH in the medium (LDH supernatant), the medium of the culture plates was transferred to eppendorfs and centrifuged at 250 x g for 4 minutes.

In both cases, samples aliquots (25 µL) were mixed with 50 µL lactate dehydrogenase assay mixture (LDH assay substrate, cofactor and dye solutions), and incubated for 20 minutes at room temperature in the dark. Absorbance was determined at λ = 492 nm on a ELISA reader (Denley, model Wellscan WS 050). The amount of LDH leakage to the medium was normalized by total LDH, and calculated as follows:

LDH leakage = LDH supernatant ÷ LDH total.

Percentage of inhibition of cell viability/proliferation in heat-treated cultures (%) was calculated as follows:

% = (LDH leakage from control cultures - LDH leakage from heat-treated cultures) x 100.

3.4.2 Number of adherent cells

B16-F10 melanoma cells and 4T1 mammary tumour cells were cultured (5×10^3 $cellcm^{-2}$) in 75 cm^2 culture flakes, in the conditions described above. Control and heat-treated cultures were tested for the number of adherent cells at defined points throughout the culture period. Cells were enzymatically released as described before and counted using a haemocytometer.

3.4.3 Mechanism of cell death

B16-F10 melanoma cells and 4T1 mammary tumour cells were cultured (5×10^3 $cellcm^{-2}$) in 90 mm culture plates. The amount of apoptotic cells was determined with TACS™ Annexin V-FITC Apoptosis Detection *Kit* (R&D Systems). This assay uses Annexin-V-FITC conjugates for detection of cell surface changes that occur early in the apoptotic process. Annexin-V is an anticoagulant protein that preferentially binds negatively charged phospholipids. Early in the apoptotic process, phospholipid asymmetry is disrupted leading to the exposure of phosphatidylserine on the outer leaflet of the cytoplasmic membrane (Fadock *et al.*, 1992; Meers and Mealy, 1993; Tait and Gibson, 1992). Propidium iodide (PI) binds to DNA molecules of cells. The combination of annexin V-FITC and propidium iodide allows the differentiation between early apoptotic cells (annexin V-FITC positive), late apoptotic cells (annexin V-FITC and propidium iodide positive), necrotic cells (propidium iodide positive) and viable cells (unstained).

Adherent cells were enzymatically detached with trypsin, transferred to a falcon and centrifuged for 7 min at 500 g. Cells were resuspended in PBS with 1% of BSA, and counted in a haemocytometer. An aliquot of 2 x 10^6 $cellml^{-1}$ was transferred to a clean falcon and centrifuged for 7 min at 500g. The supernatant was removed and 50 µL Annexin-V incubation reagent (5 µL 10x binding buffer, 5 µL PI, 0.5 µL Annexin-V-FITC, 39.5 µL dH2O) was added to the pellet. Samples were incubated for 20 minutes, in the dark, at room temperature. After this period 500 µL of 1x Binding buffer was added to samples, and proceed with the flow cytometry analysis.

3.4.4 Cell morphology and F-actin cytoskeleton labelling

B16-F10 melanoma cells and 4T1 mammary tumour cells were cultured in 30 mm culture plates (5 x 10^3 $cellcm^{-2}$) in standard culture conditions. Control cultures and heat-treated cultures were fixed at defined time points, labelled for F-actin cytoskeleton and nucleus and observed by confocal laser scanning microscopy (CLSM).

For the fixation process, the medium was removed and the cells were washed twice with warm PBS. Cells were fixed with 1 mL of formaldehyde (methanol free), and left for 15 min. Formaldehyde (methanol free) was removed, and cells were washed twice with PBS. Cells were maintained at 4ºC in PBS until labelling.

For labelling, cells were permeabilized with 0.1% triton in PBS, for 5 min. The solution was removed and cells were washed with PBS. A solution of 10 $mgmL^{-1}$ of BSA with 1 $µgmL^{-1}$ RNAse in PBS was added and left in incubation for 1 h. The solution was removed, and a solution of 5 UmL^{-1} Alexa Fluor® 647-Phalloidin (Invitrogen, Barcelona, Spain) was added and cells were incubated for 20 min in the dark. Cells were washed with PBS, and a solution of propidium iodide (10 μgmL^{-1} in PBS; Sigma-Aldrich, St. Louis, MO) was added. Cells were incubated for 10 min in the dark, at room temperature. Cells were washed with PBS, and maintained in a mounting medium (20 mM tris pH 0.8, 0.5% N-propyl gallate, 90% glycerol) before CLSM observation. CLSM was performed in a Laser Scanning Confocal Microscope Leica SP2 AOBS SE (Leica Microsystems, Germany).

3.5 Effect of conditioned medium from hyperthermia treated cultures on cell viability/proliferation of tumour cells

3.5.1 Conditioned medium

The culture medium from tumour cell cultures submitted to hyperthermia was collected at different times after heat treatment (0, 3, 6, 24 and 48 hours after treatment). The culture medium from control cultures (maintained at 37ºC during the entire culture time) was collected at the same time-points. Both conditioned media were centrifuged for 10 min at 600 g, filtered, divided in aliquots and kept at -20ºC until use.

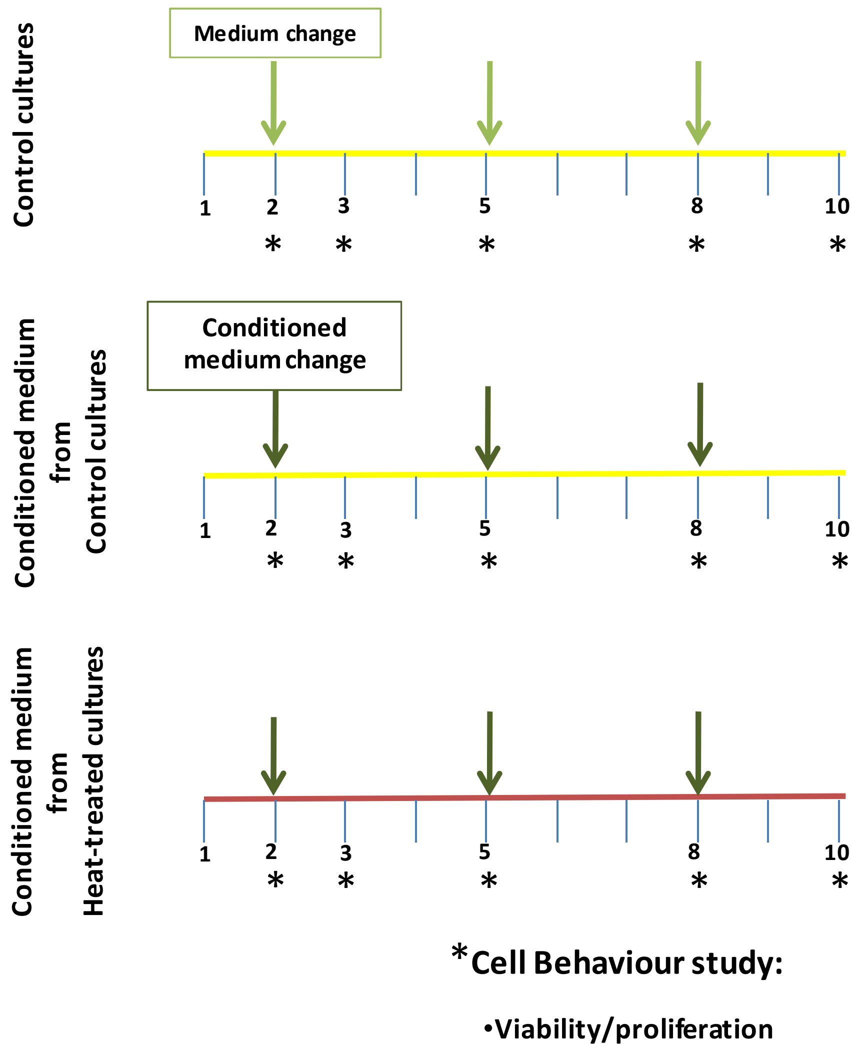

Figure 5. Diagram of the experimental protocol. : medium change. replacement of standard medium by conditioned medium.

3.5.2 Effect of conditioned medium on cell viability/proliferation of tumour cells

B16-F10 melanoma cells and 4T1 mammary tumour cells were cultured in 96-well culture plates, at a density of 10^4 cellcm^{-2}. At day 2, the medium was removed and replaced with the conditioned medium from the cultures submitted to the hyperthermia treatment, from the same cell line. Following, cells were cultured for a period up to 10 days in the presence of the conditioned medium (medium change at days 5 and 8). In parallel experiments, B16-F10 melanoma cells and 4T1 mammary tumour cells were also cultured in the presence of conditioned medium from control cultures and processed in the same way. In a separate experiment, cultures were fed with freshly prepared culture medium. Figure 5 shows a diagram of the experimental protocol used.

Cultures were characterized for cell viability/proliferation by the MTT assay. Results of the cell viability/proliferation were expressed as Absorbance (Abs).

3.6 Statistical analysis

Data are presented as mean ± SEM of four experiments. In the cell viability/proliferation assays (MTT and LDH assays) four replicas were performed in each experiment. Data were analysed with Mann Whitney U test. A p value of ≤ 0.05 was considered statistically significant.

4 Results

4.1 Effect of hyperthermia on B16-F10 melanoma cells and 4T1 mammary tumour cells

B16-F10 melanoma cells and 4T1 mammary tumour cells were seeded at a density of 5 x 10^3 cellcm^{-2}, and cultured in standard conditions. At day 5, 50 – 60% confluence, cultures were submitted to a heat treatment, at 45ºC for 30 minutes, followed by a period of incubation at 37ºC. Control cultures (incubated at 37ºC during the entire culture time) and heat-treated cultures were maintained for periods up to 14 days. The effect of the hyperthermia treatment in cell behaviour was analyzed immediately after the heat treatment and during the recovery period at 37ºC, at defined time-points, up to 9 days. Cell behaviour was assessed for cell viability/proliferation, number of adherent cells, mechanism of cell death and cellular morphology.

4.1.1 Cell viability/proliferation

Cell viability/proliferation was assessed by the MTT assay and LDH assay.

In the MTT assay, viable cells reduce the MTT salt with the formation of a formazan precipitate, which is subsequently solubilised with DMSO. The LDH assay quantifies the LDH leakage to the culture medium from damaged cells. Results are shown in Figures 6 and 7, respectively for B16-F10 melanoma cells and 4T1 mammary tumour cells.

B16-F10 melanoma cells. Figure 6 A and B shows the results observed in the MTT assay throughout the 14 days of total culture time. In control cultures, cell viability/proliferation increased significantly during the first week, and slowly afterwards. After the hyperthermia treatment, cultures showed a significant decrease in the viability/proliferation. This decrease was observed during the first 24 hours; at day 6 of the culture (24 hours after heat treatment), values were about 60% lower than those observed in control cultures. Between days 7 and 11 values decreased to 80%, and cell viability/proliferation remained very low throughout the remaining culture time.

Figures 6 C and D shows a detail of the behaviour of the culture during the first 48 hours after the heat treatment. Cell viability/proliferation decreased significantly in the first 3 hours after treatment (6.125 days of culture), increased slightly in the next hours, at 6 hours (6.25 days of culture), but began to decrease again after that period.

Results observed in the LDH assay were in line with those found in MTT assay (Figure 6 E and F). In control cultures, LDH release remained low throughout the culture time. Heated cultures showed an increase in the amount of LDH released to the medium 3 hours after the treatment, a decrease after 6 hours, followed by an increase in the remaining culture time (Figure 6 E and F).

4T1 mammary tumour cells. In control cultures, Figure 7 A (MTT assay) shows that cell viability/proliferation increased gradually until day 5, decreased slightly between day 5 and 7 and increased again until day 11. After heat treatment (day 5), cell viability/proliferation decreased gradually during the first 24 hours, and remained very low until the end of the culture period Figure 7 C and D.

LDH assay (Figure 7 E and F) provided similar results. In control cultures, LDH release remained low until day 6 and showed a small increase between days 6 and 7. In a way similar to that observed with B16-F10 melanoma cells, LDH release increased immediately after heat treatment, decreased in the following few hours (3 and 6 hours), and increased again after that period (Figure 7 E and F).

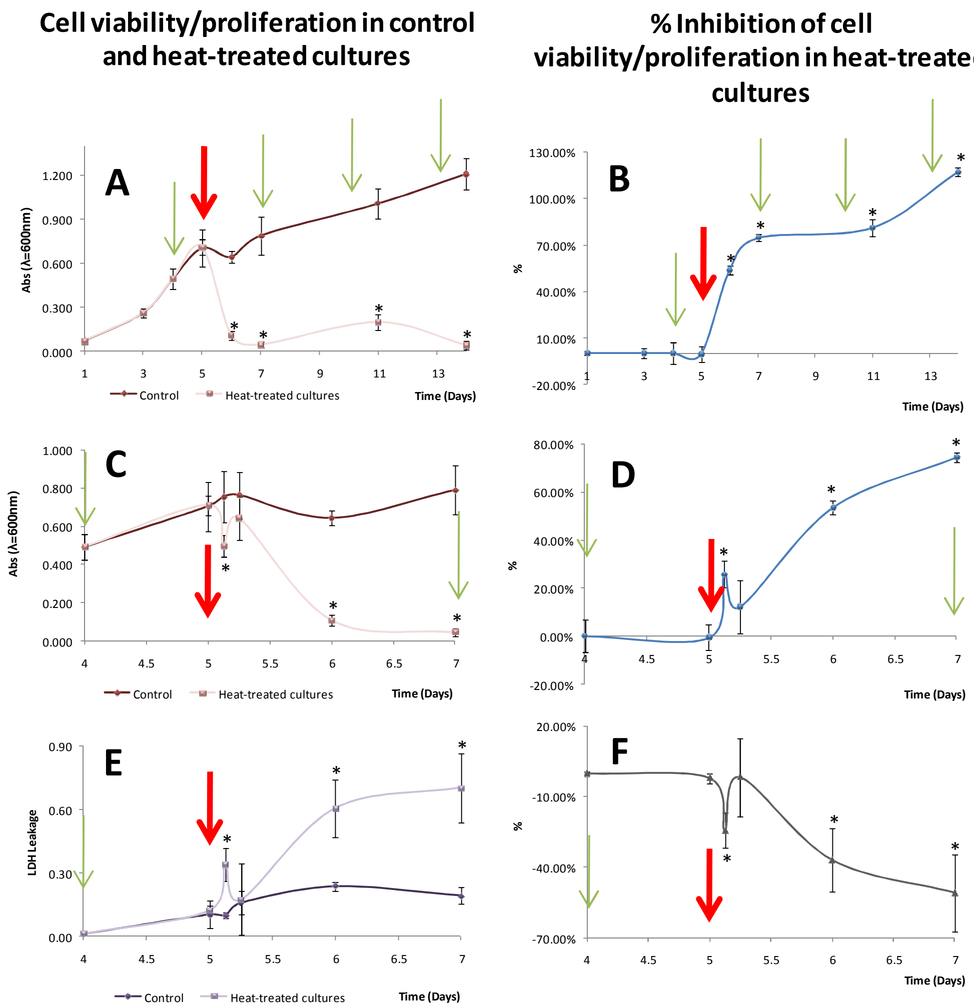

Figure 6. Effect of hyperthermia treatment in viability/proliferation of B16-F10 melanoma cells. Cultures were submitted to the heat treatment at day 5 (→), and subsequently maintained at 37ºC for further 9 days. MTT and LDH assays were performed at defined time-points. Medium was changed twice a week (→).

A – B: MTT assay, throughout the 14 day culture time.

C and D: MTT assay, detail of the cell behaviour in the first 48h after the heat treatment.

E and F: LDH assay, cell behaviour in the first 48h after the heat treatment.

A, C: Absorbance. B, D and F: % of inhibition of cell viability/proliferation in heat-treated cultures, relatively to control.

E: LDH leakage, normalized by adherent cells.

*Significantly different from control cultures (maintained at 37ºC during the 14-day culture period); p ≤ 0.05.

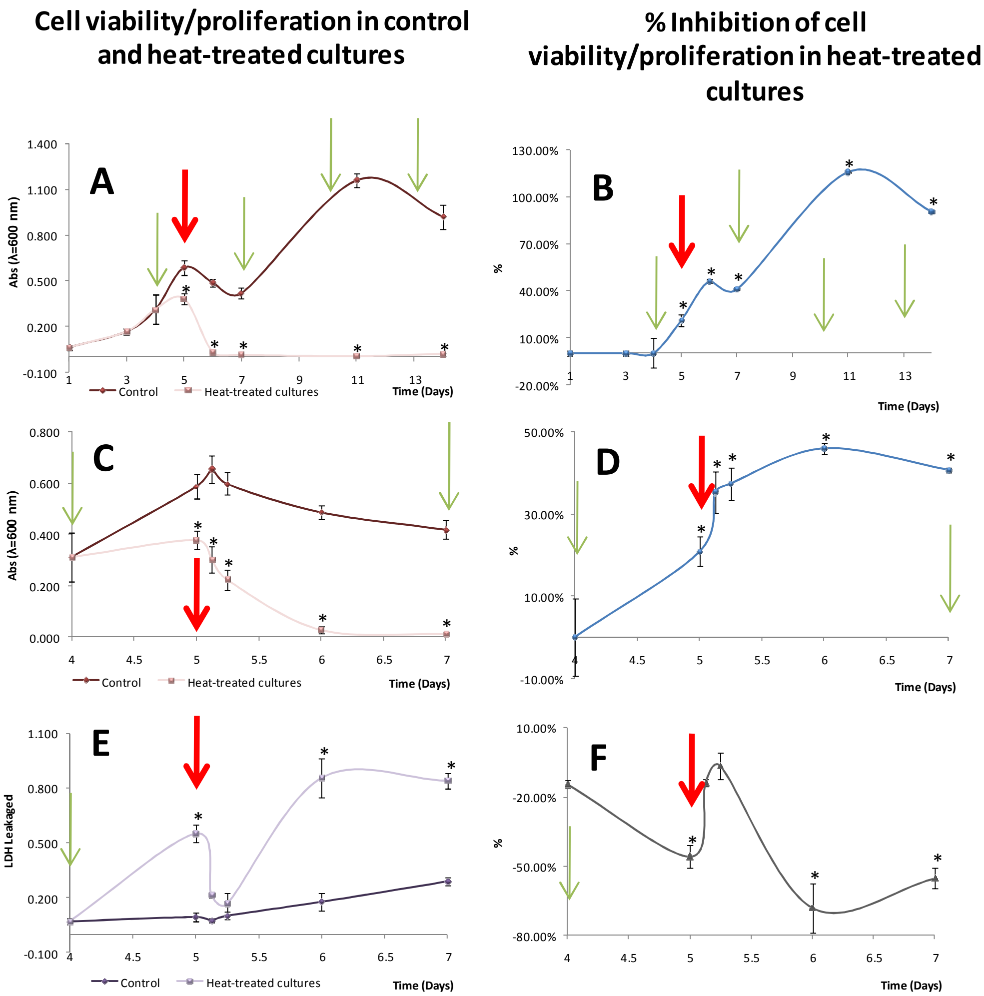

Figure 7. Effect of hyperthermia treatment in the viability/proliferation of 4T1 mammary tumour cells. Cultures were submitted to the heat treatment at day 5( ), and subsequently maintained at 37ºC for a further 9 days. MTT and LDH assays were performed at defined time-points. Medium was changed twice a week ( ).

A – B: MTT assay, throughout the 14 day culture time.

C and D: MTT assay, detail of the cell behaviour in the first 48h after the heat treatment.

E and F: LDH assay, cell behaviour in the first 48h after the heat treatment.

A, C: Absorbance. B, D and F: % of inhibition of cell viability/proliferation in heat-treated cultures, relatively to control.

E: LDH Leakage, normalized by adherent cells.

*Significantly different from control cultures (maintained at 37ºC during the 14-day culture period); p ≤ 0.05.

4.1.2 Number of adherent cells

Following hyperthermia treatment, at defined time-points (0 hours to 9 days, i.e. 5 to 14 days of culture time, respectively), cultures were tested for the number of adherent cells. Cell layer was washed twice with PBS, and the cells were enzymatically released and counted. Results are shown in Figures 8 and 9.

B16-F10 melanoma cells. Figure 8 A shows that, in control cultures, the number of adherent cells increased significantly during the first 6 days and remained approximately constant after that. The heat treatment caused a marked decrease in the cell number (Figure 8). Detail of the cell behaviour (Figure 8 B) shows that this decrease was observed in the first 3 - 6 hours after the heat treatment, and cell number remained low throughout the culture time.

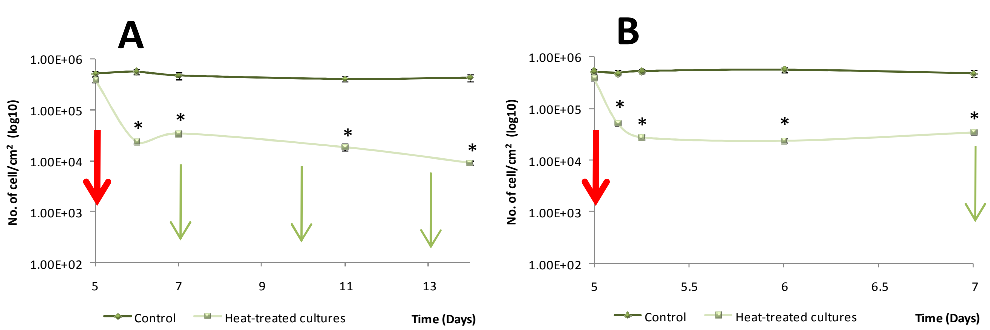

Figure 8. Effect of hyperthermia treatment in the number of adherent B16-F10 melanoma cells. Cultures were submitted to the heat treatment at day 5 ( ), and subsequently maintained at 37ºC for a further 9 days. The number of adherent cells was evaluated at defined time-points. Medium was changed twice a week ( ).

A: Cell behaviour throughout the 14-day culture time.

B: Detail of the cell behaviour in the first 48h after the heat treatment.

*Significantly different from control cultures (maintained at 37ºC during the 14-day culture period); $p \leq 0.05$.

4T1 mammary tumour cells. In control cultures, cell number increased until day 5 and remained approximately constant after that (Figure 9 A). The heat treatment caused a significant and gradual decrease in the number of adherent cells during the first 24 hours (Figure 9 B). Cell number remained low until the end of the culture period (Figure 9).

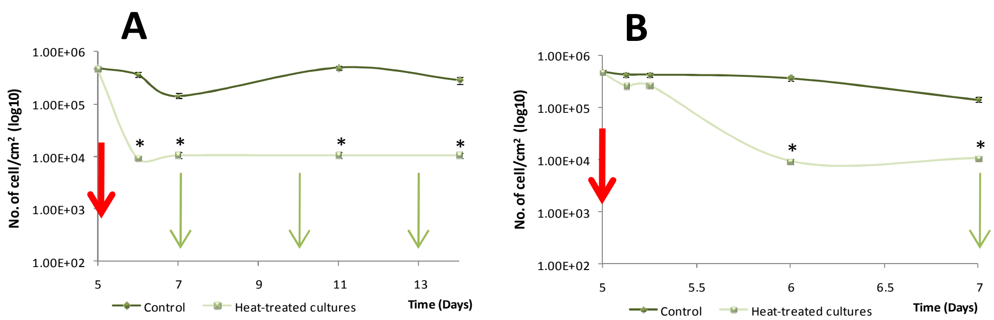

Figure 9. Effect of hyperthermia treatment in the number of adherent 4T1 mammary tumour cells. Cultures were submitted to the heat treatment at day 5 ( ), and subsequently maintained at 37ºC for a further 9 days. The number of adherent cells was evaluated at defined time-points. Medium was changed twice a week ( ).

A: Cell behaviour throughout the 14-day culture time.

B: Detail of the cell behaviour in the first 48h after the heat treatment.

*Significantly different from control cultures (maintained at 37ºC during the 14-day culture period); $p \leq 0.05$.

4.1.3 Mechanism of cell death

Evaluation of the mechanism of cell death following the hyperthermia treatment of B16-F10 melanoma cells and 4T1 mammary tumour cells was performed by flow cytometry with TACS[tm] Annexin V-FITC Apoptosis Detection kit. The combination of annexin V-FITC and propidium iodide allows the differentiation between early apoptotic cells (annexin V-FITC positive), late apoptotic cells (annexin V-FITC and propidium iodide positive), necrotic cells (propidium iodide positive) and viable cells (unstained). Results are presented in Tables 1 – 4 and Figures 10 and 11.

B16-F10 melanoma cells. Control cells showed a high percentage of live cells until day 7 (> 97%), and a small decrease afterwards (around 85% live cells). This decrease was especially due to an increase in the number of cells presenting primary apoptosis (Table 1, Figure 10). The hyperthermia treatment altered significantly this profile. In the heated cultures, the number of live cells decreased immediately after the treatment (attaining 64% at 3 hours), increased slightly in the next hours (76% at 6 hours), but, after 24h, the number of live cells was very low (20%). However, an increase in the percentage of live cells was observed again at day 7 (48h after heat treatment, 68% of live cells), followed by a decrease in the remaining culture period (around 10% of live cells). The decrease in the number of live cells was associated with an increase of secondary apoptosis until day 7, and primary apoptosis after that period (Table 2 and Figure 10). Cellular death by necrosis was not significant.

Table 1. Mechanism of cellular death in B16-F10 melanoma cells in a period of 14 days. Flow cytometry analysis of Annexin-FITC binding/PI uptake were performed at defined time-points.

Days	Live		Primary Apoptosis		Secundary apoptosis		Necrosis		No. of adherent cell/cm^2
5	98.3%	± 9.8	1.1%	± 0.1	0.3%	± 0.0	0.3%	± 0.0	$5.20 \times 10^5 \pm 4.90 \times 10^4$
5.125	97.9%	± 9.8	1.2%	± 0.1	0.5%	± 0.1	0.4%	± 0.1	$4.93 \times 10^5 \pm 4.90 \times 10^4$
5.25	98.6%	± 9.9	0.9%	± 0.1	0.2%	± 0.0	0.3%	± 0.0	$5.33 \times 10^5 \pm 5.30 \times 10^4$
6	98.9%	± 14.8	0.4%	± 0.0	0.2%	± 0.0	0.5%	± 0.1	$5.68 \times 10^5 \pm 5.70 \times 10^4$
7	96.1%	± 9.6	2.3%	± 0.2	0.7%	± 0.1	0.9%	± 0.1	$4.80 \times 10^5 \pm 7.20 \times 10^4$
11	85.7%	± 12.9	1.7%	± 0.3	11.1%	± 1.3	1.5%	± 0.2	$4.08 \times 10^5 \pm 4.00 \times 10^4$
14	84.6%	± 12.7	1.3%	± 0.1	10.8%	± 1.5	3.3%	± 0.4	$4.32 \times 10^5 \pm 6.48 \times 10^4$

Table 2. Effect of hyperthermia on mechanism of cellular death in B16-F10 melanoma cells in a period of 14 day. Cultures were submitted to the heat treatment at day 5, and subsequently maintained at 37ºC for a further 9 days. Flow cytometry analysis of Annexin-FITC binding/PI uptake were performed at defined time-points.

Days	Live			Primary Apoptosis			Secundary apoptosis			Necrosis			No. of adherent cell/cm^2
5	82.1%	± 8.2		6.6%	± 0.7	*	8.2%	± 1.0	*	3.1%	± 0.3		$3.92 \times 10^5 \pm 3.89 \times 10^4$
5.125	63.8%	± 8.9	*	13.2%	± 1.8	*	18.5%	± 2.4	*	4.5%	± 0.5	*	$5.20 \times 10^4 \pm 4.98 \times 10^3$ *
5.25	76.8%	± 9.92	*	2.2%	± 0.2		15.5%	± 1.6	*	5.5%	± 0.6	*	$2.80 \times 10^4 \pm 2.77 \times 10^3$ *
6	21.2%	± 3.2	*	0.8%	± 0.1		76.1%	± 10.7	*	1.9%	± 0.3		$2.40 \times 10^4 \pm 2.38 \times 10^3$ *
7	67.7%	± 6.8	*	0.2%	± 0.0		30.9%	± 3.7	*	1.2%	± 0.1		$3.47 \times 10^4 \pm 3.49 \times 10^3$ *
11	4.9%	± 0.6	*	94.0%	± 9.4	*	1.0%	± 0.1	*	0.1%	± 0.0		$1.87 \times 10^4 \pm 2.80 \times 10^3$ *
14	17.6%	± 2.6	*	72.7%	± 8.7	*	7.9%	± 1.0		1.8%	± 0.2		$9.33 \times 10^3 \pm 9.29 \times 10^2$ *

*Significantly different from control cultures (maintained at 37ºC during the 14-day culture period); $p \leq 0.05$.

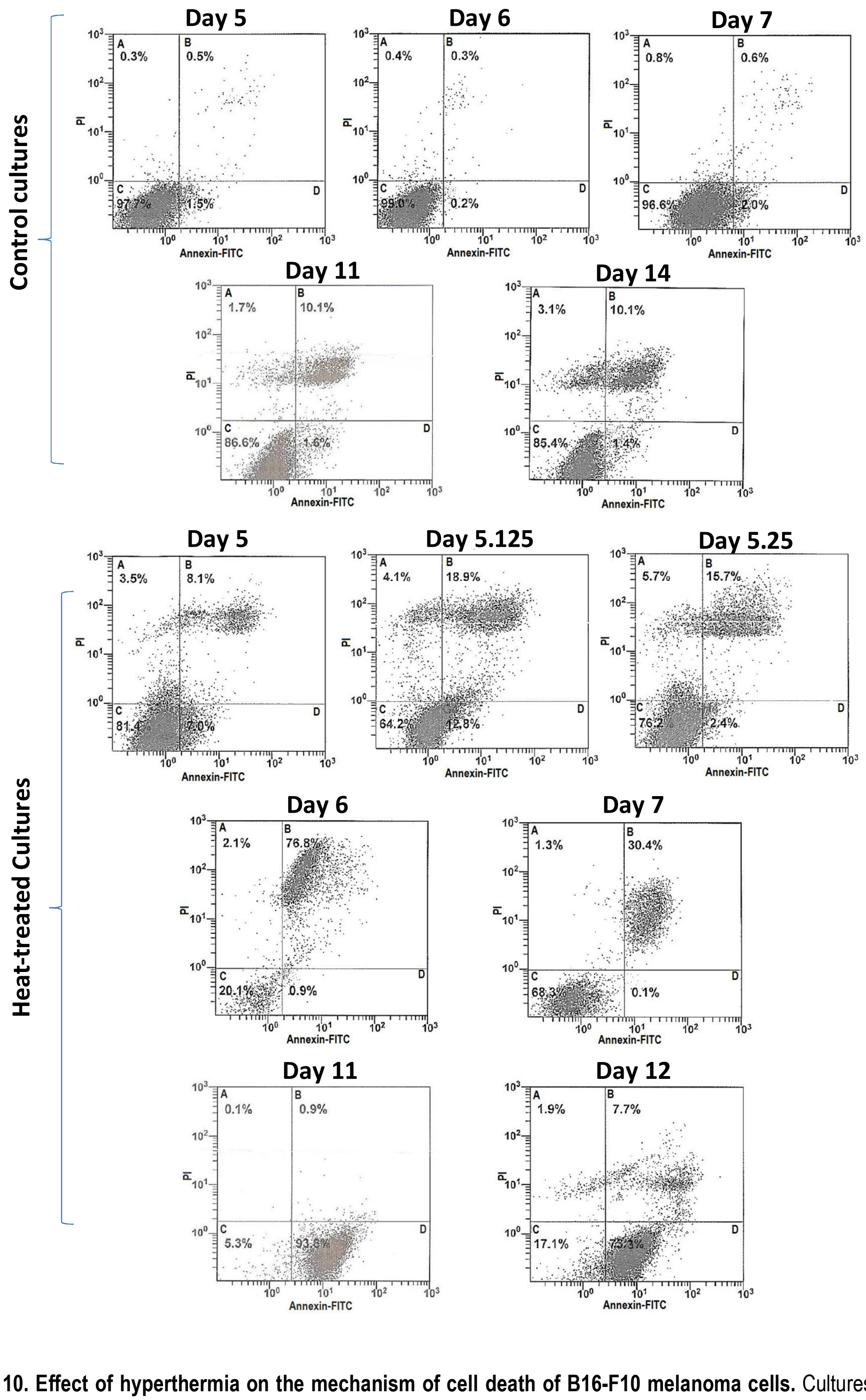

Figure 10. Effect of hyperthermia on the mechanism of cell death of B16-F10 melanoma cells. Cultures were submitted to the heat treatment at day 5, and subsequently maintained at 37ºC for a further 9 days. Flow cytometry analysis of Annexin-FITC binding/PI uptake were performed at defined time-points. Representative images of control and heat treated cultures. A: Necrosis (Annexin-/PI+); B: Secondary apoptosis (Annexin+/PI+); C: Viable cells (Annexin-/PI-); D: Primary apoptosis (Annexin+/PI-). X axis: fluorescence intensity of Annexin-FITC; Y axis: fluorescence intensity of PI.

4T1 mammary tumour cells. In control cultures, the percentage of live cells was around 80% until day 6 and decreased to values of 64% at day 7, increasing again afterwards. Cultures presented a significant percentage of secondary apoptosis (approximately 10 to 20%). Following the hyperthermia treatment, the number of live cells decreased gradually, attaining a very low value at day 7 (1.2%, 48h after heat treatment). The percentage of live cells increased between day 7 and 11, but decreased afterwards. In the heated-treated cultures, the first decrease in the percentage of live cells (during the 48h after heat treatment) was associated with an increase in secondary apoptosis, whereas the second decrease (after day 11 of culture) was mainly related with primary apoptosis. Necrosis also accounted for the cell death observed during the first 6h (9 to 14%), but remained low after that. Results are shown in Tables 3 and 4 and Figure 11.

Table 3. Mechanism of cellular death in 4T1 mammary tumour cells in a period of 14 days. Flow cytometry analysis of Annexin-FITC binding/PI uptake were performed at defined time-points.

Days	Live		Primary Apoptosis		Secundary apoptosis		Necrosis		No. of adherent cell/cm^2
5	84.3%	± 8.4	3.3%	± 0.3	10.7%	± 1.1	1.7%	± 0.2	$4.85x10^5$ ± $4.75x10^4$
5.125	84.9%	± 9.3	3.5%	± 0.4	10.6%	± 1.2	1.0%	± 0.2	$4.32x10^5$ ± $4..36x10^4$
5.25	83.4%	± 8.3	3.6%	± 0.5	11.4%	± 1.1	1.6%	± 0.2	$4.32x10^5$ ± $4.31x10^4$
6	76.9%	± 9.2	4.1%	± 0.5	16.3%	± 1.6	2.7%	± 0.3	$3.68x10^5$ ± $3.70x10^4$
7	65.1%	± 9.1	8.8%	± 0.9	23.0%	± 3.0	3.1%	± 0.3	$1.44x10^5$ ± $1.58x10^4$
11	87.6%	± 8.8	3.3%	± 0.4	7.9%	± 0.8	1.2%	± 0.1	$4.96x10^5$ ± $4.92x10^4$
14	74.2%	± 7.4	8.8%	± 1.0	15.7%	± 1.6	1.3%	± 0.2	$2.88x10^5$ ± $4.18x10^4$

Table 4. Effect of hyperthermia on mechanism of cellular death in 4T1 mammary tumour cells in a period of 14 day. Cultures were submitted to the heat treatment at day 5, and subsequently maintained at 37ºC for a further 9 days. Flow cytometry analysis of Annexin-FITC binding/PI uptake were performed at defined time-points.

Days	Live			Primary Apoptosis			Secundary apoptosis			Necrosis			No. of adherent cell/cm^2
5	74.6%	± 9.0		1.3%	± 0.2		14.5%	± 1.8		9.6%	± 0.9	*	$4.72x10^5$ ± $1.20x10^2$
5.125	55.7%	± 7.2	*	2.1%	± 0.2		28.1%	± 3.8	*	14.1%	± 1.7	*	$2.64x10^5$ ± $1.30x10^2$
5.25	54.2%	± 5.6	*	1.0%	± 0.1		32.6%	± 3.7	*	12.2%	± 1.5	*	$2.72x10^5$ ± $3.26x10^4$
6	52.8%	± 6.5	*	7.9%	± 0.9	*	33.3%	± 4.7	*	6.0%	± 0.5	*	$9.33x10^3$ ± $9.50x10^2$ *
7	1.2%	± 0.2	*	11.6%	± 1.6	*	86.9%	± 7.8	*	0.3%	± 0.0		$1.07x10^4$ ± $1.28x10^3$ *
11	74.8%	± 8.2		18.0%	± 1.8	*	7.0%	± 1.1		0.2%	± 0.0		$1.07x10^4$ ± $1.43x10^3$ *
14	40.8%	± 3.7	*	47.3%	± 5.7	*	11.5%	± 1.5		0.4%	± 0.0		$1.07x10^4$ ± $1.18x10^3$ *

*Significantly different from control cultures (maintained at 37ºC during the 14-day culture period); p ≤ 0.05.

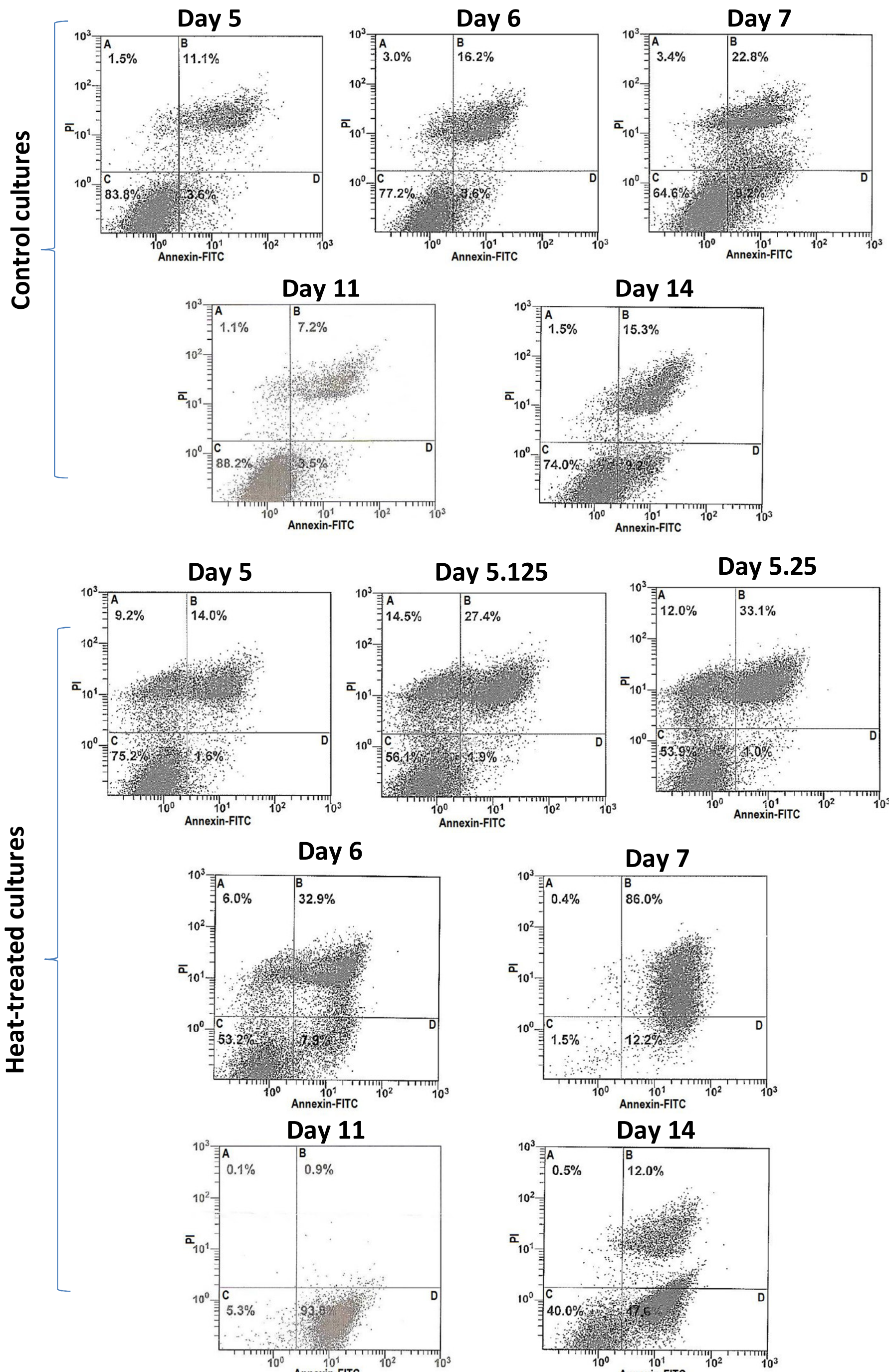

Figure 11. Effect of hyperthermia on the mechanism of cell death of 4T1 mammary tumour cells. Cultures were submitted to the heat treatment at day 5, and subsequently maintained at 37ºC for a further 9 days. Flow cytometry analysis of Annexin-FITC binding/PI uptake were performed at defined time-points. Representative images of control and heat treated cultures. A: Necrosis (Annexin-/PI+); B: Secondary apoptosis (Annexin+/PI+); C: Viable cells (Annexin-/PI-); D: Primary apoptosis (Annexin+/PI-). X axis: fluorescence intensity of Annexin-FITC; Y axis: fluorescence intensity of PI.

4.1.4 Cell morphology

Control and heat-treated cultures were stained for the F-actin cytoskeleton and nucleus and were observed by CLSM. Representative images are shown in Figure 12 and 13, respectively for B16-F10 melanoma cells and 4T1 mammary tumour cells.

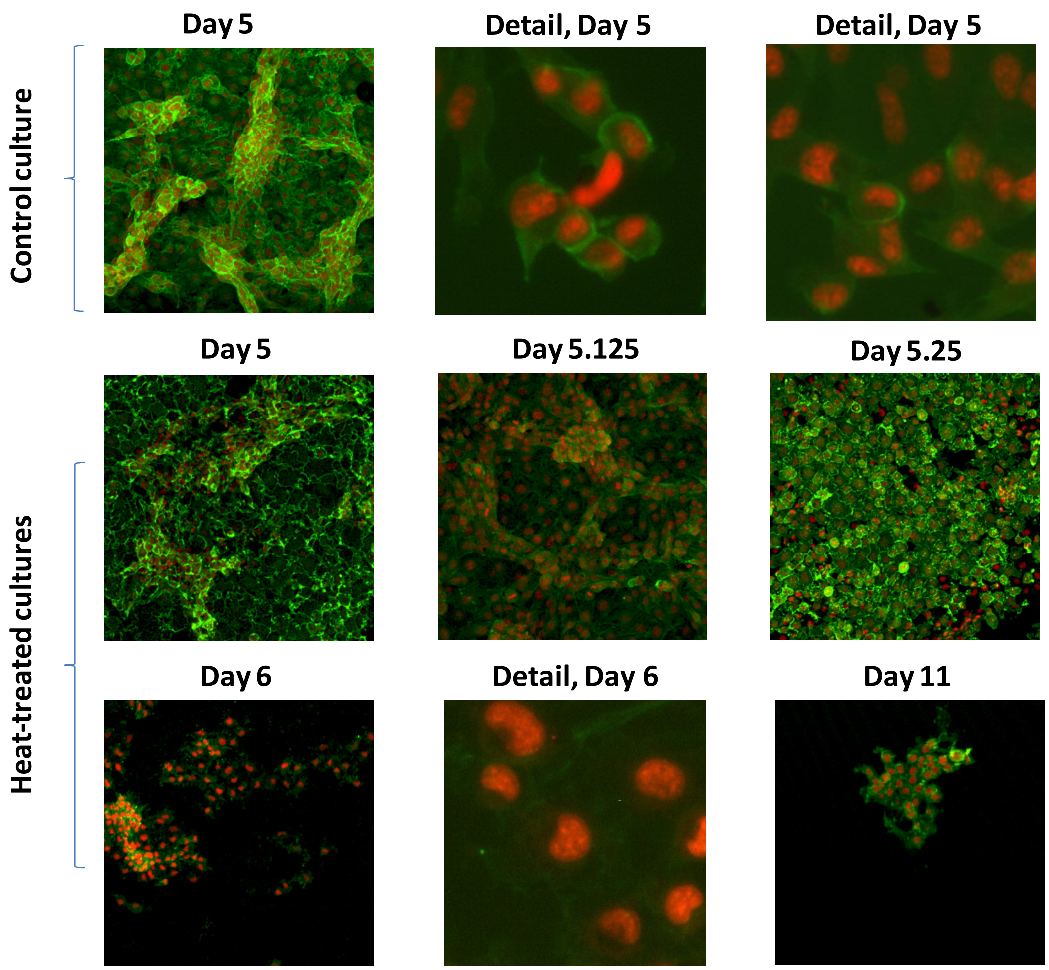

Figure 12. Effect of hyperthermia on the morphology of B16-F10 cells. Cultures were submitted to the heat treatment at day 5, and subsequently maintained at 37ºC for a further 24 hours. Morphology was accessed at defined time-points (0, 3, 6 and 24 hours after the heat treatment). Representative images of control and heat treated cultures are presented. F-actin cytoskeleton and nucleus staining. Confocal microcopy: 300x; Detail: 500x.

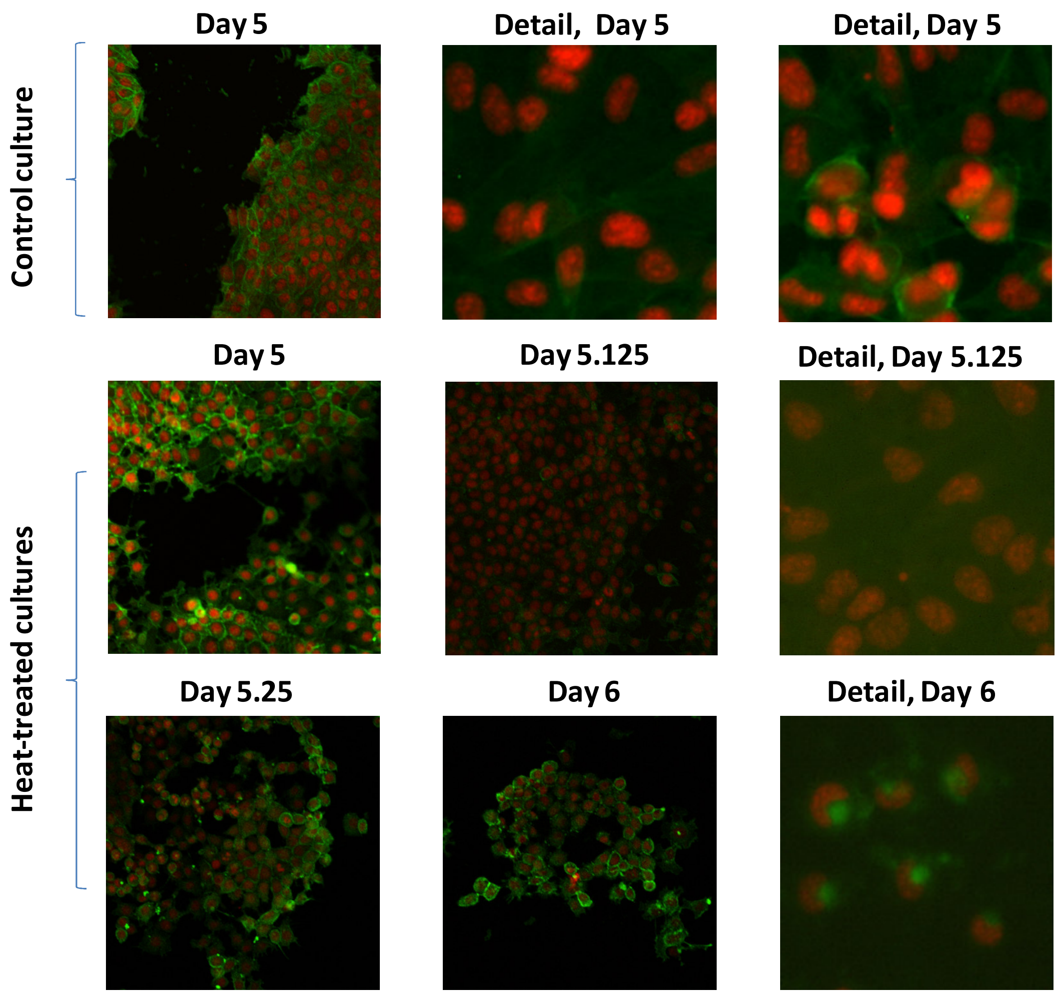

Figure 13. Effect of hyperthermia on the morphology of 4T1 mammary tumour cells. Cultures were submitted to the heat treatment at day 5, and subsequently maintained at 37ºC for a further 24 hours. Morphology was accessed at defined time-points (0, 3, 6 and 24 hours after the heat treatment). Representative images of control and heat treated cultures are presented. F-actin cytoskeleton and nucleus staining. Confocal microcopy: 400x; Detail: 500x.

Control cultures, both B16-F10 melanoma cells and 4T1 mammary tumour cells, presented an organized cell layer with extensive cell-to-cell contact and areas of higher cell density. In addition, cells displayed a well-spread F-actin cytoskeleton and a prominent nucleus. The hyperthermia treatment caused a significant alteration on this morphologic profile. In both cell lines, the effect of the heat treatment began immediately. Cultures showed a progressive loss of the organization of the cell layer and cell-to-cell contact, a decrease in the number of adherent cells and morphologic alterations characterized by rounded shape, cytoplasm shrinkage with loss of the structure of F-actin cytoskeleton and condensation of the nuclear material.

4.2 Effect of conditioned medium from hyperthermia treated cultures in the viability/proliferation of B16-F10 melanoma cells and 4T1 mammary cancer cells

The culture medium from B16-F10 melanoma and 4T1 mammary tumour cell cultures, both control and submitted to the heat treatment (45ºC, 30 min) was collected at defined time points, i.e. immediately after the treatment (0 hours) and after 3, 6, 24 and 48 hours, and was added to growing cultures of the same type. In parallel, cultures were fed with freshly prepared culture medium. Cultures were maintained for 10 days and medium was changed twice a week. Cell cultures were characterized for cell viability/proliferation by MTT assay. Results are presented in Figures 14 and 15, respectively for B16-F10 melanoma cells and 4T1 mammary tumour cells.

B16-F10 melanoma cells. Cultures maintained with freshly prepared culture medium presented a progressive increase in the viability/proliferation during the culture period. Cultures fed with conditioned medium from control cultures collected at 5, 5.125 and 5.25 days showed a significant decrease in the MTT values until day 5, but cell proliferation increased afterwards; however, maximum values were lower than those found in the cultures maintained with fresh prepared medium. Cultures fed with conditioned medium collected at day 6 and day 7 maintained a low level of viability/ proliferation during the culture period. (Figure 14).

Cultures fed with conditioned medium from heated cultures, collected immediately after the heat treatment (0 h) presented a progressive decrease in the cell growth, and from day 5 onwards MTT values were very low. However, conditioned medium from heated cultures collected 3 and 6 hours after the heat treatment did not cause significant effects compared to those observed in the cultures maintained with conditioned medium from control cultures. Conditioned medium from heated cultures collected 24 hours after the treatment caused an initial decrease in the viability/proliferation followed by a significant increase in this parameter. The presence of conditioned medium from heated cultures collected 48 hours after the treatment resulted in a similar result, although in a lower level. Results are shown in Figure 14.

4T1 mammary tumour cells. Cultures maintained in freshly prepared culture medium showed an increase in the cell proliferation throughout the incubation period (Figure 15). However, a significant decrease in the cell proliferation was observed in the cultures maintained with conditioned medium from control cultures, collected at the various time-points; this effect was more evident in the cultures fed with conditioned medium collected at day 7. A significant decrease was found from day 2 to day 3, and, after that, cells proliferated slowly, with maximum MTT values being significantly lower than those observed in the cultures fed with freshly prepared medium.

Conditioned medium from the heated cultures caused a significant decrease in cell viability/proliferation of 4T1 mammary tumour cells. This inhibitory effect was evident with the conditioned medium collected at 0, 3 and 6 hours. However, the presence of conditioned medium collected 24 hours after the heat treatment caused an increase in viability/proliferation when compared with that observed in the cultures fed with conditioned medium from control cultures collected at the same time. Conditioned medium collected from heated cultures 48 hours after the treatment caused a significant inhibitory effect. Results are displayed in Figure 15.

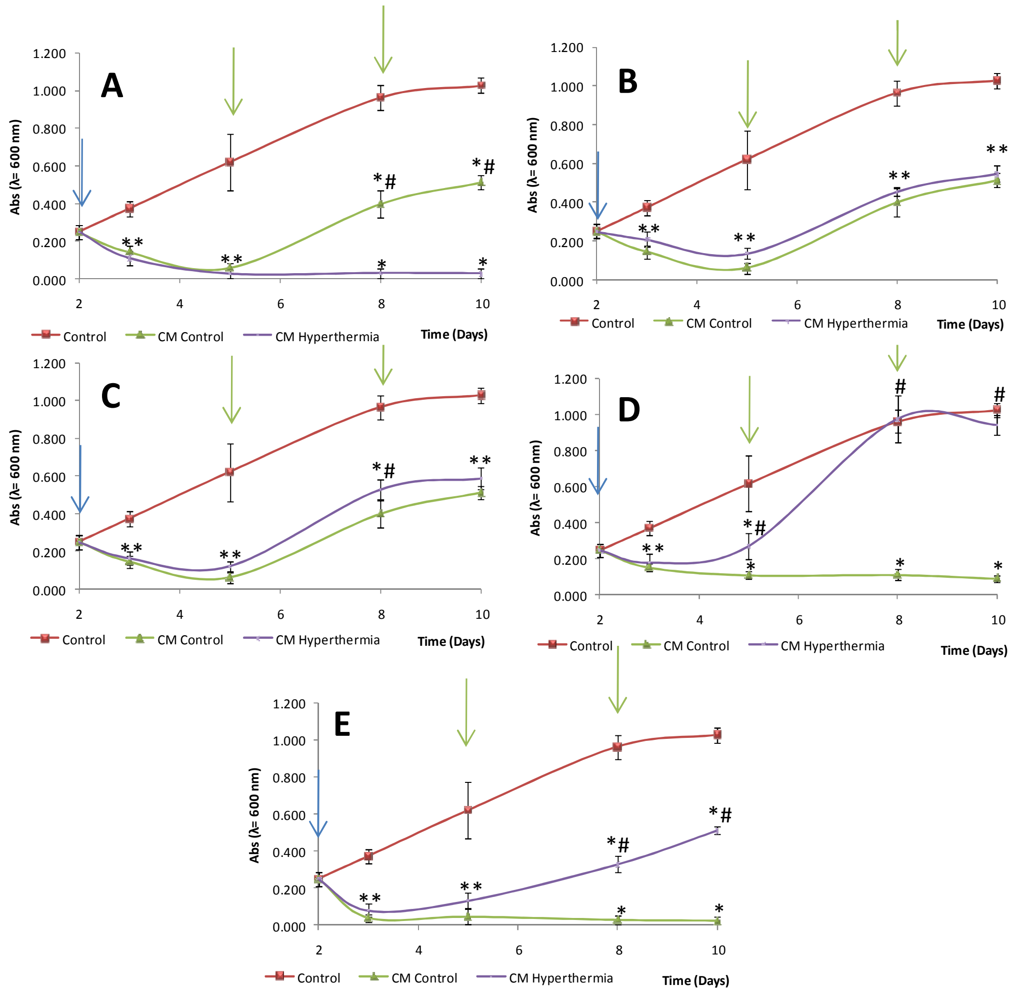

Figure 14. Effect of conditioned medium from heat-treated B16-F10 melanoma cell cultures in the viability/proliferation of B16-F10 melanoma cells. Cultures were maintained for 10 days, and conditioned medium was added at day 2 (⟶) and replaced twice a week (⟶). Cultures were also maintained in the presence of conditioned medium from control cultures and also performed with freshly prepared medium. MTT was performed at defined time-points.

Cultures fed with conditioned medium collected at 0 h (A), 3 h (B), 6 h (C), 24 h (D) and 48 h (E) after the heat treatment in heat-treated cultures (CM hyperthermia) or with conditioned medium from cultures that were used as control, collected at the same time-points (CM Control); Control: cultures fed with freshly prepared culture medium.

*Significantly different from control cultures (maintained in freshly prepared culture medium); $p \leq 0.05$.

#Significantly different from cultures maintained in conditioned medium from control cultures; $p \leq 0.05$.

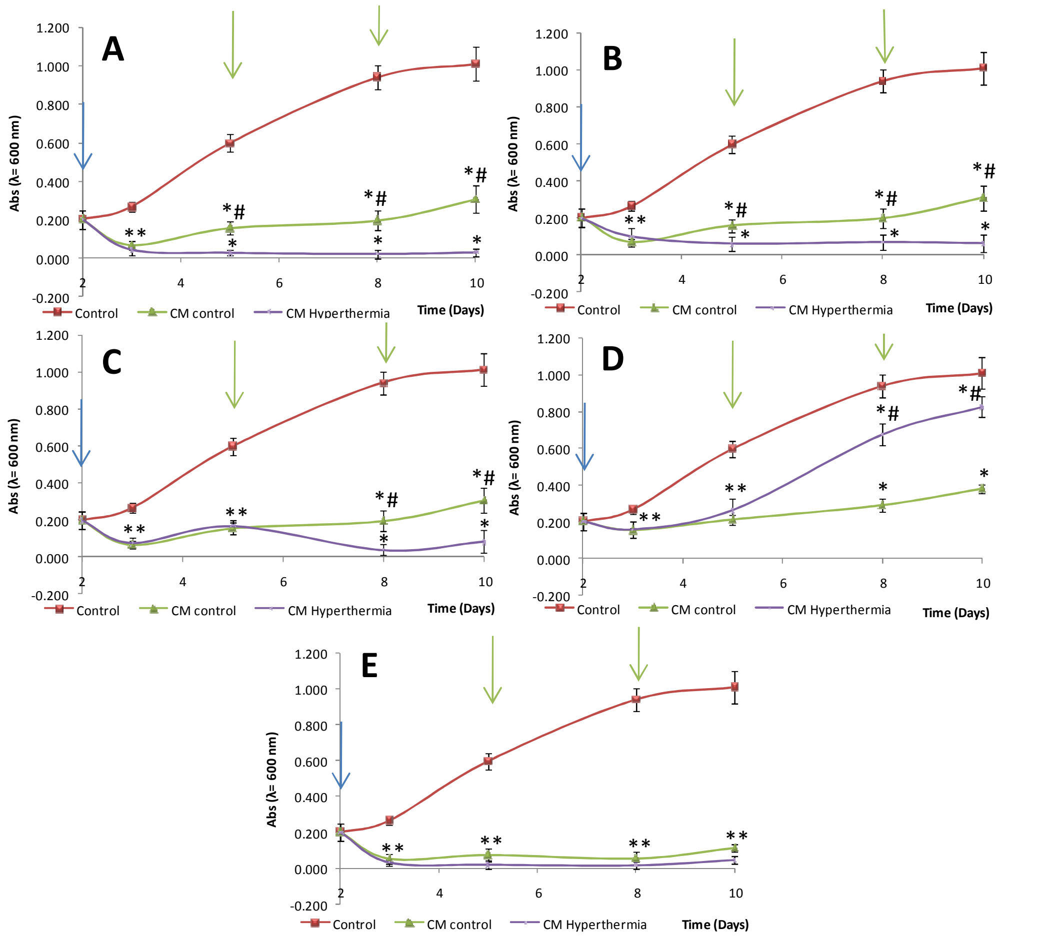

Figure 15. Effect of conditioned medium from heat-treated 4T1 mammary tumour cell cultures in the viability/proliferation of 4T1 mammary tumour cells. Cultures were maintained for 10 days, and conditioned medium was added at day 2 ( ) and replaced twice a week ( ). Cultures were also maintained in the presence of conditioned medium from control cultures and also performed with freshly prepared medium. MTT was performed at defined time-points.

Cultures fed with conditioned medium collected at 0 h (A), 3 h (B), 6 h (C), 24 h (D) and 48 h (E) after the heat treatment in heat-treated cultures (CM hyperthermia) or with conditioned medium from cultures that were used as control, collected at the same time-points (CM Control); Control: cultures fed with freshly prepared culture medium.

*Significantly different from control cultures (maintained in freshly prepared culture medium); $p \leq 0.05$.

#Significantly different from cultures maintained in conditioned medium from control cultures); $p \leq 0.05$.

5 Discussion

Hyperthermia is a cancer therapy based on the knowledge that heat can kill cells after a temperature breakpoint, above fever temperature (Vertrees *et al.*, 2007). This therapy can be used alone or in conjugation with other established therapies, as radiotherapy or chemotherapy. Although it's already being used as cancer therapy, there still is a lot to uncover about the effects that are critical to the treatment and destruction of cancer cells and cancerigenous tissues.

The effect of hyperthermia on cells and tissues has been studied *in vitro*, *in vivo* and in some clinical trials.

In a clinical trial, patients with metastatic bone tumour that have received a heat treatment at 43ºC (10 times, 15 minutes per day) achieved a good outcome compared with patients only operated, and a similar outcome compared to patients operated and submitted to radiotherapy (Matsumine *et al.*, 2007).

Using the same temperature of 43ºC, some *in vivo* studies have shown the benefits of this therapy. As Yanase *et al.* (1998) described for F344 rats bearing glioma, a hyperthermia treatment of 30 minutes is capable of reducing the tumour and is even possible to induce a reaction of the immune system leading to the destruction of the tumour metastasis. Kaway and is coworkers (2005) have reported a similar effect when F344 rats bearing prostate cancer were treated at temperature of 45ºC for 30 minutes.

In vitro studies have also demonstrated a variety of effects of hyperthermia in the cells. It has been reported effects at all levels in the cells, namely functional mechanisms, alterations in gene expression and proteins and at last cell death by necrosis or apoptosis (Vertrees *et al*, 2007; Hildebrandt *et al.,* 2002, Zhou et al., 2007). Although there are few exceptions, most works show promising results in the cure of cancer, but the effects are dependent of temperature, time of exposure and the type of cells used.

In this work, two cells lines, B16-F10 melanoma cells and 4T1 mammary tumour cells, cultured at a density of 5 x 10^3 cellcm^{-2}, were submitted to hyperthermia, at 45ºC for 30 minutes. Effects in cell viability/proliferation, number of adherent cells, mechanism of cellular death and cell morphology were assessed in specific time points, in a period of 14 days. Control cultures were kept at 37ºC for the same culture period. The results showed alterations in these parameters in both cell lines after hyperthermia treatment, but there were some differences in the way heat affected these two cells lines.

B16-F10 cell viability/proliferation was most affected in the first 24 hours after treatment. Within this period, the MTT assay showed that heat shock reaction was first noticed after 3 hours by a decrease in viability/proliferation and, although cells began to recover afterwards (at 6 hours), they were not able to recover to control levels. Effect in cell viability/proliferation was also assessed by LDH leakage to medium, corresponding to cells with alterations in the cellular membrane that led to release of cellular content to the medium. As observed with MTT assay, the heat shock was first noticed at 3 hours and between 3 and 6 hours cells had a recovery. After that, cells began a new cycle of degradation. The number of adherent cells was also affected by hyperthermia. This effect was observed immediately after the heat treatment and was most noticed at 3 hours, with cell number being significantly lower than that of control cultures. Analysis of the mechanism of cellular death by flow cytometry showed, in the first 48 hours, an augmentation of cellular death by apoptosis (especially at 24 hours, by secondary apoptosis) and some necrosis. This was followed by a second period of cellular degradation and death by primary apoptosis. These deleterious effects of heat treatment were also observed in the morphology of cells, by presenting morphologic alterations such as rounded shape and cytoplasm shrinkage; these alterations were progressive and irreversible.

From these observations it was possible to find important periods of the heat shock reaction in B16-F10 melanoma cells. After treatment, there was an acute effect on cells submitted to hyperthermia, leading to the damage of cellular membrane, decrease of viability/proliferation, alterations in cell morphology and augmentation of cellular death. After this period, cells that had non-lethal damages began to recover, showing a decrease in LDH leakage to the medium, augmentation of cell activity and increase in the percentage of live cells. After this, cells began a period of cellular degradation, possibly because of structural and functional damages that inhibit cell viability/proliferation.

4T1 mammary tumour cells showed a more pronounced damage effect after hyperthermia treatment. The effect on viability/proliferation was observed immediately after heat treatment. In the MTT assay, viability/proliferation of heated-treated cells immediately started to decrease, showing a gradual effect with time after the first heat shock reaction. The heat shock reaction was also observed regarding LDH leakage to the medium, with a similar effect to that observed in the MTT assay. In this case, LDH leakage was observed immediately after the heat treatment, which was followed by a recovery of the cells, but, after 6 hours, the concentration of LDH in the medium began to increase. The number of adherent cells present after hyperthermia was most affected 24 hours after heat treatment and then was followed by a period of stabilization. Cellular death started to increase from the time of heat treatment. Within 48 h after the treatment, it was possible to observe an augmentation in cell death by secondary apoptosis and also an increase in necrosis. After this period, the percentage of live cells started to increase. However, it is worth to mention that the number of adherent cells was significantly lower than that of control, and the remaining cells presented low viability. In the later stages of the culture period cell death was accomplished by primary apoptosis.

From the results described it was possible to find one important period for the heat shock reaction in 4T1 mammary tumour cells. The hyperthermia effects were more drastic than those observed with B16-F10 melanoma cells, but the first 24 hours were still critical for the observation of hyperthermia effect. Immediately after treatment, a decrease in viability/proliferation and increased of LDH leakage was observed, and although LDH leakage decreased afterwards, cells did not recover again. In this case, cellular number was most affected after 24 hours. Degradation of these cells was evident, as also observed in morphologic alterations and augmentation of cellular death.

Results of this study showed that hyperthermia treatment at 45ºC has affected B16-F10 melanoma cells and 4T1 mammary tumour cells differently in the first hours of the treatment. But in the period that follows, cells reacted in a similar way. This method induced cellular death by several mechanisms. In the first hours after heat treatment (24 – 48 hours), cellular destruction was accomplished mostly by secondary apoptosis and some necrosis (especially for 4T1 mammary tumour cells). The remaining cells began to die later (several days after the heat treatment) mostly by primary apoptosis. At this stage, number of adherent cells and cell viability were very low. In both cell lines, these deleterious effects were accompanied with significant morphologic alterations, as cell shrinkage and features characteristics of apoptosis. These observations suggest that the heat treatment caused rapid and intense cell damage, evidenced by the increase in cellular death by secondary apoptosis in the first 24 – 48 hours after hyperthermia, limiting the observation of primary apoptosis. However, the heat treatment also appeared to induce other cellular alterations because, several days after the heat treatment, primary apoptosis was the main mechanism of cell death.

There are some previous studies regarding hyperthermia at elevated temperatures, as 45ºC, in several cell lines.

Sharif-Khatibi and coworkers (2007) described alterations in K562 erythroleukaemia cells, at 45ºC for 20 or 40 minutes, like decrease in viability and cellular growth in a period of 5 days after heat treatment. They observed an increase in cell death by primary apoptosis (approximately 21%) 24 hours after heat treatment and secondary apoptosis was only observed after 72 hours.

A different observation was described by Babsky *et al.* (2005) reporting that a temperature of 45ºC, for 30 minutes, did not affect viability ratio but affected the plating efficiency in RIF-1 cells.

Another study has shown that heating B16-F10 melanoma cells at 46ºC for 30 min can in fact affect viability, leading to a great amount of cell death (60%) immediately after treatment (Oliveira-Filho *et al.,* 1997); in this work, authors heated cells in a suspension of PBS-EDTA which is expected to result in a higher cell damage compared to the heat treatment of an adherent cell layer.

Human umbilical vein endothelial cells (HUVECs) also showed to be affected by hyperthermia at 45ºC. When these cells were submitted to hyperthermia at 45ºC for 60 minutes, viability was similar to control immediately after, but 120 minutes later viability was reduced by 50% (Fukao *et al.* 2000).

Also, several studies reported hyperthermia induced alterations in morphology as cell shrinkage and detachment, and also alterations characteristics of apoptotic cell death. These effects were described in human prostatic stromal cells when submitted to a treatment of 47ºC for 1 hour, which were found to progress with time of culture (Brehmer and Svensson, 2000). These morphological alterations are described by some authors as characteristics of apoptotic cell death (Creagh *et al*., 2000¸Majno and Joris, 1995).

At lower temperatures (43ºC), hyperthermia treatment did not affect cellular behaviour in the same way than that found at 45ºC (Sharif-Khatibi *et al*., 2007). It has been proposed that augmentation of HSP expression that occurs at 43 ºC protects cells from apoptosis. HSP can protect cells from alterations in protein structures, or to repair and restore the structure and functional integrity of damaged proteins, when cells are submitted to a stress as hyperthermia (Ito *et al.,* 2006; Manjili *et al.,* 2002; Schmitt *et al.,* 2007). These authors also showed that at high temperatures, as 45ºC, there were no alterations on HSP concentration, in the first 8 hours after treatment, so cells failed to protect themselves and undergo apoptosis (Sharifi-Khatibi *et al.* 2007). Milani and coworkers (2005) showed that, in melanoma cells, heated at 45ºC, the HSP are only induced 15 hours after treatment, having is higher level 48 hours after treatment. Nijhuis *et al.* (2008) reported an augmentation of HSP70 levels in hematopoietic cells submitted to hyperthermia treatment at 42ºC for 1 hour. They also showed that cells with lower levels of p53 have a decreased susceptibility to undergo apoptosis. p53 is a protein that acts as a transcriptional factor involved in cell cycle regulation, a loss of its function through mutation results in genetic instability and an impaired induction of apoptosis (Nijhuis *et al*, 2008; Van dyke, 2007).

The previous mentioned studies show that, for a similar heat treatment, the effects of hyperthermia in cell viability and mechanism of cell death are dependent on the cell line. Also, the heat treatment affects cells in a temperature and time manner. Thus, comparison of the results of the present work with those reported in the literature is difficult. However, in line with the previous studies, in this work, B16-F10 melanoma cells and 4T1 mammary tumour cells, submitted to the same experimental protocol, showed differences in the hyperthermia induced effects, which might be related with a different expression profile of HSP70 and p53 molecules following the heat treatment (Beere, 2005, Nijhuis *et al.,* 2008;

Takahashi, 2001). Further studies would need to be done to test this hypothesis in the hyperthermia outcome in this work.

As mentioned above, the hyperthermia treatment of B16-F10 melanoma cells and 4T1 mammary tumour cells caused cell damage and, ultimately, cell death. These events led to extravasation of cellular content to the medium and, eventually, the presence of cytotoxic compounds. Also, the heat treatment induces the expression of several relevant molecules that influence cell behaviour. To test this hypothesis, the conditioned medium from heat-treated cultures, collected at several time-points after the heat treatment (0 to 48 hours), was used to feed cell cultures of the same tumour line. In addition, these cultures were also performed in the presence of conditioned medium from cultures that served as control of the heat-treated cultures. Cell viability/proliferation of tumour cell cultures fed with the conditioned media was compared to that of similar cultures fed with freshly prepared medium.

Both B16-F10 melanoma and 4T1 mammary tumour cell cultures fed with conditioned media from control cultures presented decreased cell viability/proliferation in a time-dependent manner, compared to that of cultures maintained with freshly prepared medium. Several factors might account for this observation, namely, consumption of nutrients and augmentation of toxic substances resulting from the cell metabolic activities during the culture time. As a result, a significant decrease in the cell growth was observed in the few days following the addition of the conditioned medium. However, afterwards, cells adapted to this medium environment and cell proliferation increased, but maximum MTT values were significantly lower than those found in the cultures supplemented with freshly prepared medium.

Conditioned medium from B16-F10 melanoma and 4T1 mammary tumour cell cultures submitted to hyperthermia caused significant effects on the viability proliferation of growing B16-F10 and 4T1 mammary tumour cells, respectively. However, conditioned medium collected at various time-points after the heat treatment (0 to 48 hours) showed different effects.

In B16-F10 melanoma cells, the supplementation with conditioned medium collected immediately after the heat treatment (0 hours) caused an evident decrease in cell viability/proliferation, compared to that found in the cultures supplemented with conditioned medium from control cultures collected at the same time-point. This inhibitory effect was not observed when B16-F10 melanoma cell cultures were fed with conditioned medium from heated cultures collected at 3 and 6 hours after the heat treatment; in these conditions, cell viability/proliferation was similar to that found in the cultures supplemented with the culture medium from control cultures. Conditioned media collected 24 and 48 hours after the treatment increased cellular viability/proliferation (especially that collected at 24 hours). The initial inhibitory effect might be related to the presence of toxic compounds in the conditioned medium liberated from damaged cells. On the other hand, the augmentation of viability observed when conditioned medium collected at 24 and 48 hours was added to the cell cultures could be caused by exhaustion/inactivation of the toxic compounds that have damaged cells until that point; it is also possible that some substance in the medium is leading to this augmentation, eventually synthesised in response to the heat treatment.

Conditioned medium from 4T1 mammary tumour cells submitted to hyperthermia showed that medium collected 0, 3, 6 and 48 hours after the heat treatment had the ability of decreasing cell viability/proliferation of these tumour cells, compared to that observed in the cultures fed with conditioned medium from control cultures. This decrease might be an effect of cell content leakage to the medium. This cell line was more susceptive to hyperthermia, showing various deleterious effects at these time-points after heat treatment, as evidenced by LDH leakage. However, it was also possible to observe an increase in viability in the presence of conditioned medium collected 24 hours after treatment, which, as explained before, it could be an effect of exhaustion of toxic compounds or presence of some substance leading to this augmentation.

As mentioned before, cell death can lead to leakage of cell content to medium, leading to an increase of its toxicity; this would eventually lead to deleterious effects in the remaining viable cells. One possible factor that leads to these alterations is the formation of free radicals and reactive oxygen species (ROS). Free radicals are highly reactive substances that can interact with cellular molecules, leading to cellular damage. A stress as the one resulting from hyperthermia can lead to liberation of free radicals to the medium (Halliwell *et al.,* 1999; Thannickal and Fanburgo, 2000). Flanagan and coworkers (1998) have described that hyperthermia treatment, 45ºC for 20 minutes, increases the flux of free radicals in cells. This report also found evidences that the increase of free radicals and ROS and the resultant oxidative stress may mediate heat-induced cellular damage. Grasso *et al* (2003) also reported an increase of ROS after a heat treatment of human fibroblasts at 42ºC for 40 minutes. Although it is in a lower temperature they also comported that hyperthermia can lead to formation of this reactive species. The same authors reported that the formation of ROS after hyperthermia contributes to growth arrest and less to apoptosis activation. In this way, according to the observations of these studies, the second period of cellular degradation observed in the present work regarding B16-F10 melanoma cells and 4T1 mammary tumour cells is probability mediated by toxicity of the culture medium.

As conclusion, hyperthermia can affect cells in two different ways, directly and indirectly. After heat shock, cells begin to decrease viability, start losing their normal morphology and eventually die by apoptosis and/or necrosis. These are the direct outcomes from heating cells at 45ºC for 30 minutes. Further, the cellular death leads to a second, indirect effect of hyperthermia. Cell viability decreases because of augmentation of toxic substances in the medium, like free radicals. So, hyperthermia effect in cells is a complex phenomenon, it affects all function of cells and cell death can be induced by various pathways. The results presented here show some evidences of the potential benefits of hyperthermia usage in treatment of cancer.

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6 Conclusions

Hyperthermia is a therapy based on heat induction as a method to treat cancer. Previous reports have recognised that the outcome from heating cell cultures are dependent of multiple factors, as time and temperature of exposure, cell line and period to access the heat shock reaction. In this study, two cell lines, B16-F10 melanoma cells and 4T1 mammary tumour cells, were submitted to a hyperthermia treatment, 45ºC for 30 minutes.

After heat treatment there was an acute effect on B16-F10 melanoma cells submitted to hyperthermia treatment. The heat shock reaction was first noticed 3 hours after heat treatment and led to damage of cellular membrane, decrease of viability/proliferation, alterations in cell morphology and augmentation of cellular death (specially by secondary apoptosis). Although cells started to recover, structural and functional damages led to a second period of cellular degradation and cellular death by primary apoptosis, a few days after heat treatment.

Hyperthermia effects were more drastic on 4T1 mammary tumour cells. For this cell line the heat shock reaction was noticed immediately after the treatment and the first 24 hours were critical to the observation of hyperthermia effect. Degradation of these cells was evident, as observed in morphologic alterations and augmentation of cellular death especially by secondary apoptosis and also necrosis, and later by primary apoptosis.

Hyperthermia treatment used in this work has affected B16-F10 melanoma cells and 4T1 mammary tumour cells differently in the first hours of the treatment. But in the period that follows, cells reacted in a similar way. This method induced cellular death by several mechanisms, from secondary apoptosis and necrosis (after the treatment) to primary apoptosis (last days of culture).

In line with the previous studies, in this work, B16-F10 melanoma cells and 4T1 mammary tumour cells, submitted to the same experimental protocol, showed differences in the hyperthermia induced effects, which might be related with a different expression profile of molecular chaperones involved on apoptosis pathways (HSP70 and p53 molecules) following the heat treatment, because these molecules are known to be important in the hyperthermia outcome on cellular behaviour.

Hyperthermia treatment of B16-F10 melanoma cells and 4T1 mammary tumour cells caused cell damage and, ultimately, cell death. These events led to extravasation of cellular content to the medium and, eventually, the presence of cytotoxic compounds. Also, the heat treatment is known to induce the expression of several relevant molecules that influence cell behaviour.

Conditioned medium from B16-F10 melanoma and 4T1 mammary tumour cell cultures submitted to hyperthermia caused significant effects on the viability/proliferation of growing B16-F10 and 4T1 mammary tumour cells, respectively. However, conditioned medium collected at various time-points after the heat treatment (0 to 48 hours) showed different effects. A decrease in cell viability/proliferation was observed in the presence of conditioned medium collected at 0 hours (B16-F10 melanoma cell line) and collected at 0, 3, 6 and 48 hours (4T1 mammary tumour cell line), which might be related to the presence of toxic compounds in the conditioned medium released from damaged cells. One possible factor that leads to these alterations is the formation of free radicals and reactive oxygen species (ROS) that can cause cellular damage. In this way, according to the observations of these studies, the second period of cellular degradation observed in the present work regarding B16-F10 melanoma cells and 4T1 mammary tumour cells is probability mediated by toxicity of the culture medium.

As conclusion, hyperthermia can affect cells in two different ways, directly and indirectly. After heat shock, cells begin to decrease viability, start losing their normal morphology and eventually die by apoptosis and/or necrosis. These are the direct outcomes from heating cells at 45ºC for 30 minutes. Further, the cellular death leads to a second, indirect effect of hyperthermia. Cell viability decreases because of augmentation of toxic substances in the medium, like free radicals. So, hyperthermia effect in cells is a complex phenomenon, it affects all function of cells and cell death can be induced by various pathways. The results presented here show some evidences of the potential benefits of hyperthermia usage in treatment of cancer.

7 Future Perspectives

Further studies are necessary to better understand the results presented in this work. Molecules such as HSP70 and p53 are important factors in the outcome of hyperthermia and the alterations in cellular concentration of these proteins need to be tested in both cell lines. Further studies are needed to prove that alterations in toxicity of the medium are mediated by increase of free radicals after hyperthermia treatment. Also, normal cells should be tested to attest if they are less susceptive to this hyperthermia treatment, at 45ºC.

As different temperatures will result in different outcomes, it is also necessary to test this treatment at lower temperatures, normally used in hyperthermia treatment. Also, different times of exposure should be tested to analyse the hypothesis of a time-dependent effect of hyperthermia.

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