Cell to Cell Interactions Influence Sensitivity of Liver Cell Lines during Hyperthermia

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Abstract. *Background: Thermal cancer therapy is used for hepatocellular carcinoma treatment. In this study we investigated the effect of hyperthermia on liver cells and compared data of our different cell culture fibrosis models (transwell vs. co-culture model). Materials and Methods: The cell lines HepG2 and LX-1 were seeded in different numbers in transwells to simulate different grades of fibrosis and then heated from 55˚C to 85˚C for different time spans. Thereafter, metabolic activity was measured. Results: Heating at 65˚C showed that the greater the number of LX-1 cells treated together with HepG2 cells the lower the metabolic activity of HepG2 cells was. Compared to our previous co-culture study, there were significantly different results in cell survival from 55˚C to 75˚C. Conclusion: The co-culture fibrosis model is more physiological than the transwell model because it allows a higher seeding density and a higher degree of cell to cell interactions. Therefore, it is more efficient for investigating the effect of hyperthermia on liver cells.*

Hepatocellular carcinoma (HCC) accounts for 85% to 90% of primary liver cancer cases and is the fifth most common malignant tumour in humans (1). Thermal cancer therapy is a therapeutic option for HCC patients who cannot be treated with resection or transplantation due to the advanced stage of the tumour and the underlying liver cirrhosis at the time of diagnosis (2-9). These thermal therapy techniques are commonly performed to destroy tumour tissue by using high temperatures. Blood flow, blood pressure, as well as the composition of the liver tissue (amount of fibrosis), are known to crucially impact on therapy outcome (10-14).

Most hyperthermic studies deal with temperatures ranging from 42-46˚C to investigate the influence of heat shock and thermotolerance (15). However, there is lack of knowledge on the heat sensitivity of cells at temperatures ranging from 55˚C to 100˚C which are currently used during thermal cancer therapy (5). In the literature, a cell culture study of Obara *et al*. (16) supports the need for investigations in this sector. In their analysis of heat sensitivity of three different HCC cell lines, they showed that a too low temperature, which does not induce cell death, can in fact support further malignant transformation of HCC tumour cells.

Our group investigated the metabolic activity of the HCC cell line HepG2 and the hepatic stellate cell line LX-1 after exposure to hyperthermia at high temperatures (55˚C to 100˚C). To simulate *in vivo* fibrosis, we established a co-culture model of these two cell lines. In this model, we demonstrated that the heat resistance of the co-cultures was dependent on the percentage of LX-1 cells (17). The aim of the present study was to gain more information on the metabolic activity of each single cell line during hyperthermia and determine if the survival of each of these cell lines is influenced by the other. For this reason, we established a transwell cell culture model.

Materials and Methods

Cell culture and heating experiments. The human HCC cell line HepG2 (ATCC# HB-8062; Rockville, MD, USA) was cultivated at 37˚C in minimum essential medium (MEM; Invitrogen, Austria) containing additional 10% fetal bovine serum (FBS; PAA, Austria) and 1% penicillin/streptomycin (P/S; PAA) in a humified atmosphere (5% CO_2). The hepatic stellate cell line LX-1 (kindly provided by Professor Friedman, Mount Sinai School of Medicine, NY, USA (18)) was cultivated in Dulbecco's modified Eagle's medium (DMEM containing 4.5 g/l D-glucose; Invitrogen) containing additional 10% FBS, 1% P/S and 1% L-glutamine (PAA) in a humified atmosphere (5% CO_2). For all experiments, MEM with 10% FBS and 1% P/S was used.

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Key Words: Hyperthermia, thermal cancer therapy, hepatic stellate cells, fibrosis, HCC, transwell, co-culture.

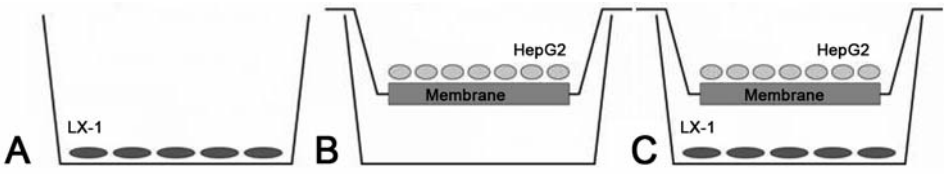

Figure 1. *Cells prepared for experiments: LX-1 cells were placed into the lower compartment and incubated with tumour growth factor- beta 1 for 18 hours overnight (A); HepG2 cells were grown on the membrane of the upper compartment (B). For the heating experiment and the follow -up culture, the cells remained together in one well (C).*

Different numbers of LX-1 cells were seeded in 12-well plates (Corning, NY, USA) and treated with 2.5 ng/ml recombinant human tumor growth factor-beta 1 (TGF-β1; Biovision, Mountain View, CA, USA) for 18 h (Figure 1A) according to the literature (17-19). Additionally, HepG2 cells were seeded onto a transwell insert (Corning, NY, USA) with a lower compartment containing only culture medium (Figure 1B).

In order to simulate different grades of fibrosis, different numbers of HepG2 and LX-1 cells were seeded in the compartments: 1×10^5 cells (100%), 7.5×10^4 cells (75%), 5×10^4 cells (50%) and 2.5×10^4 cells (25%). For heating and cultivating afterwards, the compartments were combined to gain 100% of cells (*e.g.* 75% HepG2 and 25% LX-1 cells; Figure 1C).

Immediately before heating, the culture supernatant was withdrawn and the inserts (containing HepG2 cells) were put into the wells (containing LX-1 cells) in order to represent the desired amount of fibrosis (Figure 1C). Subsequently, 1.5 ml of preheated medium was added to the 12-well plate and 0.5 ml to the transwell insert; the plates were immediately transferred into a heating cabinet. After heating at desired temperature (55˚C, 65˚C, 75˚C or 85˚C for 5 min, 10 min or 15 min), the warm medium was exchanged for 37˚C growth medium. One part of the plates was immediately used for metabolic activity testing; the other part was cultured for 24 and 48 h (LX-1 and HepG2 cells together in one well) then analysis of metabolic activity was performed as described below.

Metabolic activity testing. For the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) test (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Austria) the compartments were separated and the different cell lines were separately incubated with 500 µl medium containing MTS reagent (1:5 dilution) for 4 h. Thereafter, 2× 120 µl of the supernatant were transferred into a 96 well plate and measurement was performed using a SpectraMax 384 (Molecular Devices, Sunnyvale, CA, USA) exactly as described by the manufacturer.

Statistical analysis. For statistical analysis, we calculated the average from four different wells from each experiment; each experiment was repeated four times. Data were normalized to a control value at 37˚C. Statistical analyses and graphs were created with GraphPad Prism version 5.00 software for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Comparison of the data was performed using one-way ANOVA with Tukey's *post hoc* test (SPSS 18; PASW Statistics 18, IBM, NY, USA). Comparison of co-culture data published in a previous study (17) and transwell experiments was performed using Lavene test, *t*-test and Mann-Whitney *U*-test (SPSS 18). A value of $p<0.05$ was considered as being statistically significant.

Results

Results after heating in transwells. Heating HepG2 and LX-1 cells at 55˚C in different compartments of transwells showed no significant differences in metabolic activity (data not shown). Heat exposure at 65˚C for 5 minutes (Figure 2) led to the death of nearly all HepG2 cells and about 50% of LX-1 cells over 48 h. When HepG2 cells were cultivated in transwells with no or few (25%) LX-1 cells, they showed better metabolic activity compared to cultivation with a higher LX-1 cell density (50% and 75%). Immediately after heating at 65˚C for 10 min metabolic activity of 25% HepG2 cells was significantly lower ($p<0.05$) compared to LX-1 cells; after 48 h culture in transwells, all HepG2 cells died. Heating at 65˚C for 15 min induced cell death in nearly all HepG2 cells ($p<0.001$); LX-1 cells died within the following 24 h. Heating at 75˚C and 85˚C showed survival of a small number (0-8%) of LX-1 cells immediately after heating, which also lost metabolic activity within the first 24 h (data not shown).

Comparison of co-culture and transwell experiments. Cell survival was different comparing co-culture (17) and transwell experiments. Generally, cells cultivated together in one well seem to be more heat resistant compared to cells cultivated in transwells (Figure 3), except at a temperature of 55˚C and 15 min of heating. In this experimental setting, after 48 h of cell culture a higher number of cells survived in transwells than in co-cultures ($p<0.001$). Heating at 65˚C for 5 min led to a significantly higher metabolic activity of cells in co-culture compared to those in transwells ($p<0.01$ to $p<0.001$) after 24 and 48 h. Immediately after heat exposure for 10 min ($p<0.001$), as well as for 15 min ($p<0.01$ to $p<0.001$), significantly more viable cells were detected in co-cultures comparing the two experimental setups. Forty-eight hours after heating for 5 min at 75˚C, significantly higher metabolic activity was measured in co-cultures compared to transwells ($p<0.001$). Cells heated in transwells died immediately after hyperthermia; in contrast in co-culture experiments, cells exhibited a constant viability of 75-80%. In contrast to transwells, where no living cells were detected after heating at high temperatures (75˚C for 10 min and 85˚C for 5 min), cells in co-cultures immediately, and after 48 h, had a survival rate up to 25%.

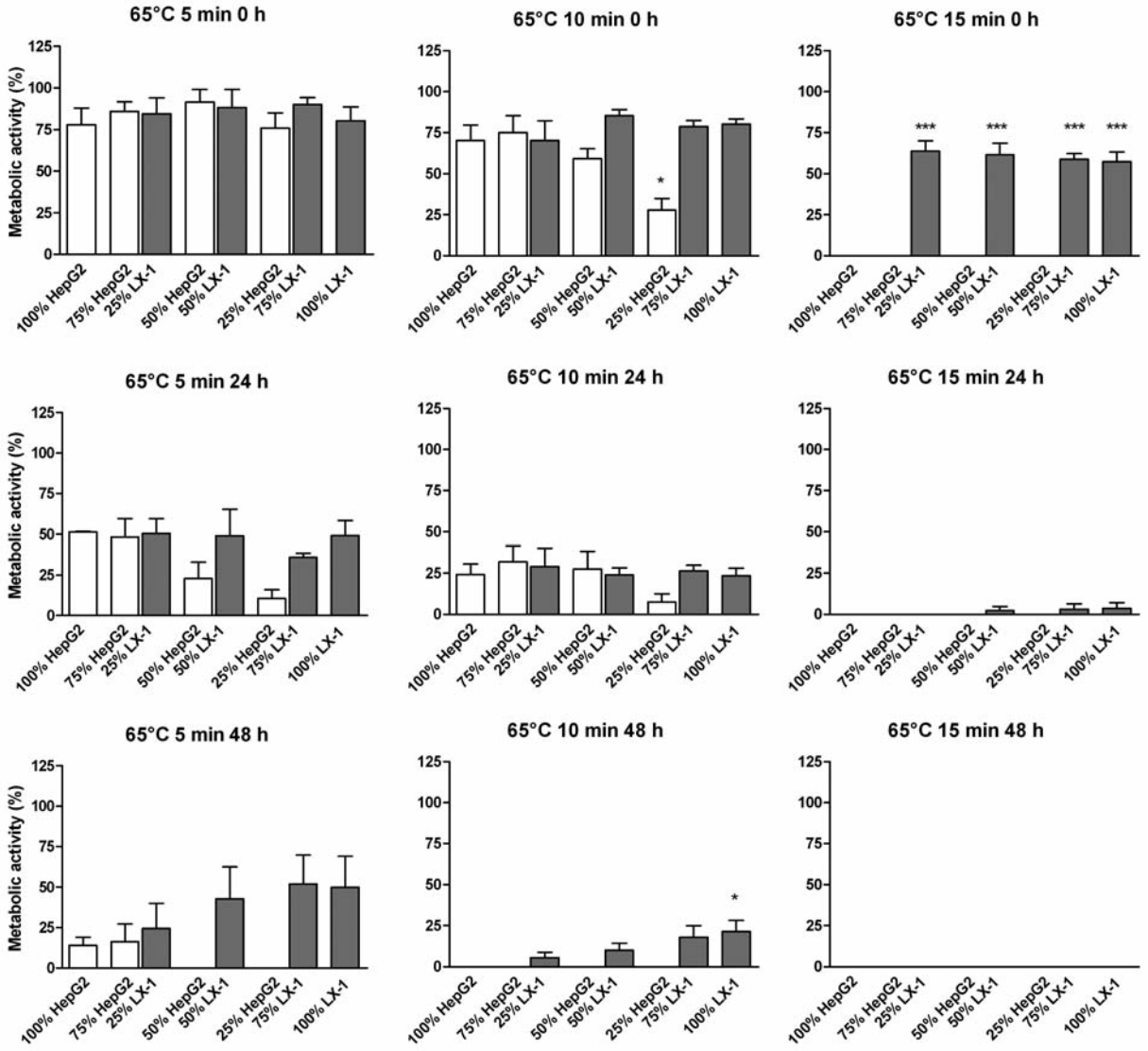

Figure 2. *Metabolic activity of HepG2 and LX-1 cells after heating at 65˚C in transwells. Our results showed that the higher the density of LX-1 cells in the transwells, the lower the metabolic activity of the HepG2 cells was. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test (*p<0.05, **p<0.001).*

Discussion

Hyperthermic treatment seems to be a valuable treatment option for patients suffering from HCC (2-9), but there is lack of knowledge on a cellular level. Therefore, we performed a study on metabolic activity after heating two different cell lines (HepG2 and LX-1) and focused on the effect on cells separated by membranes (transwell) in comparison to our previously published data on co-cultures (17).

In contrast to our previous co-culture study, we measured the metabolic activity for each cell line individually to gain more information about the heat sensitivity of the different cell lines. The cell line LX-1 seems to be more heat tolerant compared to HepG2. After heating the cells in the different compartments at 65˚C, analysis of metabolic activity revealed that the more LX-1 cells are cultured together with HepG2 cells in transwells, the lower the metabolic activity of HepG2 cells becomes (Figure 2). This phenomenon can be explained by the fact that HepG2 cells are more heat sensitive compared to LX-1 cells (17) and when they are seeded in a smaller proportion (25% of HepG2 cells indicates a number of only 2.5×10^4 cells) to represent a higher grade of fibrosis, the heat causes more damage due to the low seeding density and the lack of cell to cell

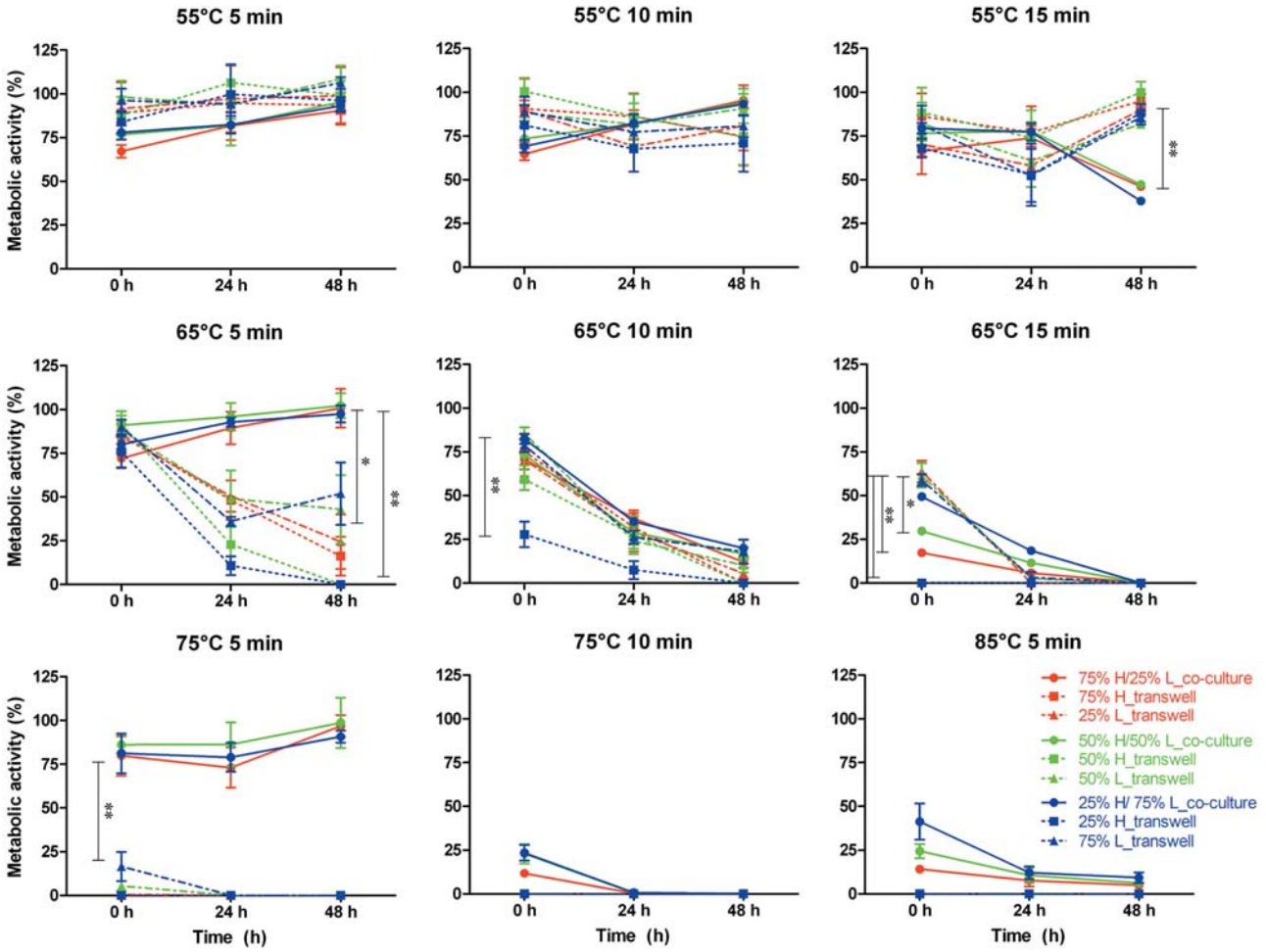

Figure 3. *Comparing co-culture and transwell heating experiments. Heating HepG2 (H) and LX-1 (L) cells in co-cultures led to a better cell survival than cultivating them in transwells (*p<0.01, **p<0.001).*

interaction. In contrast, the metabolic activity of LX-1 cells is not affected by the seeding density.

Additionally, we found significantly different cell survival rates comparing the results of the co-culture (17) and the transwell experiments. Generally, after heating at 55˚C, compared to transwell cultures cells cultivated in co-cultures showed only marginal differences in terms of metabolic activity after short-term heat exposure (5 min, 10 min) (Figure 3). Heat exposure at 55˚C for 15 min led to different survival rates comparing after 48 h co-culture to transwell experiments. This point may be critical for cell survival and minimal deviation in heat exposure could lead to a completely different result. It is possible that the cooling due to the additional membrane in transwells biased the measured cell viability.

Heat exposure at 65˚C or higher showed clear significant differences comparing co-culture and transwell experiments. Generally, cells cultivated together in one well seem to be more heat resistant in contrast to those in transwells. This may be due to the lack of cell to cell interactions, which can lead to an increased sensitivity to hyperthermia. Furthermore, simulation of fibrosis in the co-culture model matches physiological liver tissue much better than the transwell model does. Taking into account all the results of our two different cell culture experiments on hyperthermia, we are convinced that there is enough evidence that the co-culture model seems to be a more adequate model for analysing the effects of hyperthermia on liver cell lines as well as different grades of fibrosis/cirrhosis.

In conclusion, this study provides insights into the influence of hyperthermia-simulating temperatures reached during thermal cancer therapy in cell cultures. Within these experiments, the evidence that cell to cell interactions, which are rather more likely in the more physiological co-culture fibrosis model, are crucial for investigating the heat

sensitivity of liver cell lines. Our cell culture trial demonstrates that temperature and the grade of fibrosis in the treated liver may be important factors influencing the outcome after thermal cancer therapy, because we showed the higher the percentage of fibrosis (the greater the numbers of LX-1 cells) the more heat and/or temperature is necessary to induce cell death, which is very important for patient survival, as well as in planning the treatment of liver tumours using thermal cancer therapy.

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