

Doctoral thesis

**Impact of Hyperthermia caused by Radiofrequency
Ablation on Liver Cell Lines and Tissue**

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Table of Contents

Abstract.....	1
Zusammenfassung	2
1 Introduction.....	3
1.1 Hepatocellular Carcinoma.....	3
1.1.1 Liver fibrogenesis	3
1.1.2 Diagnosis and therapy	6
1.2 Other types of liver neoplasm and metastases	8
1.2.1 Colorectal metastases	8
1.2.2 Uveal melanoma metastases.....	8
1.2.3 Breast metastases.....	8
1.2.4 Neuroendocrine metastases	9
1.2.5 Cholangiocarcinoma.....	9
1.3 Therapy options for HCC	10
1.3.1 Surgical resection and liver transplantation	10
1.3.2 Transcatheter arterial chemoembolization	11
1.3.3 Percutaneous ethanol injection therapy	11
1.3.4 Percutaneous microwave coagulation therapy	11
1.3.5 Laser-induced thermotherapy.....	12
1.3.6 Radiofrequency ablation.....	12
1.3.6.1 Factors influencing RFA outcome.....	13
1.3.6.2 Changes in liver lesion after RFA	14
1.4 Wound healing in healthy tissue.....	15
1.4.1 The inflammatory phase	16
1.4.2 The proliferative phase	16
1.4.3 The maturation phase	17
1.5 Aim	18

2	Material & Methods.....	20
2.1	Co-culture experiments with MRC-5 and HepG2	20
2.1.1	Metabolic activity testing.....	20
2.1.2	Viability staining.....	21
2.2	Co-culture experiments with LX-1 and HepG2.....	21
2.2.1	Metabolic activity testing.....	21
2.2.2	Viability staining.....	22
2.3	Transwell experiment.....	22
2.3.1	Metabolic activity testing.....	24
2.4	Statistical analysis.....	25
2.5	Flow Cytometry	25
2.5.1	Intracellular staining protocol	25
2.5.2	Heating protocol.....	26
2.5.3	Staining for Flow Cytometry measurement.....	26
2.5.4	Statistical analyses	27
2.6	Animal experiments.....	29
2.6.1	Keeping of animals	29
2.6.2	Anaesthesia	29
2.6.3	Radiofrequency ablation	30
2.6.4	Large scale sections	34
2.6.4.1	Histological staining	34
2.6.5	Standard paraffin sections.....	35
2.6.5.1	Immunohistochemical staining protocols	36
2.6.5.2	Antibodies and other reagents.....	41
3	Results	42
3.1	Co-culture experiments with MRC-5 and HepG2 cells	42
3.1.1	Cell survival after temperature exposure for 5min	45
3.1.2	Cell survival after temperature exposure for 10 min	47

3.1.3	Cell survival after temperature exposure for 15 minutes	48
3.2	Co-culture experiments with LX-1 and HepG2 cells	49
3.2.1	Cell survival after temperature exposure for 5 min.....	50
3.2.2	Cell survival after temperature exposure for 10 min.....	55
3.2.3	Cell survival after temperature exposure for 15 minutes	56
3.3	Comparison of MRC-5 and LX-1 cells in co-culture experiments.....	57
3.4	Transwell experiments	58
3.4.1	Cell survival after temperature exposure for 5 minutes	61
3.4.2	Cell survival after temperature exposure for 10 minutes	64
3.4.3	Cell survival after temperature exposure for 15 minutes	65
3.5	Comparison of co-culture and transwell experiments	66
3.6	Flow Cytometry	68
3.7	Animal experiments.....	73
3.7.1	Overview	73
3.7.2	Immunohistochemical staining.....	75
3.7.3	Influence of vessels on RFA outcome.....	81
4	Discussion.....	83
4.1	Cell culture experiments	84
4.2	Porcine liver tissue after RFA	90
4.3	Conclusion	94
5	Acknowledgement.....	96
6	Appendix.....	97
6.1	Figures.....	97
6.2	Tables	98

6.3	Abbreviations.....	99
6.4	References	100
6.5	Staining protocols for immunohistochemistry.....	112
6.6	Publications	118

Abstract

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumour, with a high mortality rate. Radiofrequency ablation (RFA) is a therapeutic option for patients, which cannot be treated with resection or transplantation. We aimed to characterise the effect of RFA on HCC and healthy liver tissue in this study with the main focus on cell culture experiments simulating different grades of fibrosis and cirrhosis. Moreover, we evaluated the effect of RFA in a porcine model to monitor changes in and around the affected area.

In our cell culture experiments, we established an *in vitro* fibrosis model to investigate the heat sensitivity and cell survival on different cell lines. MRC-5 or LX-1 cells were heated with HepG2 cells in co-culture and transwells from 55°C to 100°C for different time spans. Thereafter, we measured the metabolic activity immediately, after 24 h and 48 h using MTS test to assess how many cells survived heating. Furthermore, we investigated LX-1 and HepG2 cells after heat exposure using Flow Cytometry to distinguish between apoptosis and necrosis. In the large animal model we performed RFA in pigs and euthanized them after different time spans (0d, 1d, 2d, 1w, 2 w, 1m, 2m, 3m). At each time point we gain one large and one standard paraffin block, which were sliced and stained for CAB, MB, Caspase 3, Hsp70, Ki-67, Calprotectin, GFAP and α -SMA to investigate the changes in the transition zone after RFA.

We demonstrated that fibrosis has a wide influence on the shape and the size of a RFA lesion. To prevent further tumour growth or alteration a temperature of at least 75°C has to be reached in the border area.

High temperatures cause a necrotic and thermally fixed lesion, which appears to resist tissue repair and resolution. In the transition zone, apoptotic cells are replaced by fibroblasts producing a fibrous capsule over time. This capsule acts as inflammatory border and causes mainly foreign body reaction, which leads to a chronic local inflammation.

Generally, RFA causes acute inflammatory reaction due to wounding of liver tissue. A capsule is formed around the ablation zone, which reacts as inflammatory border and causes foreign body reaction. If the tumour cells are killed completely within the RFA, the ablated area will vanish over years and will cause no further complications.

Zusammenfassung

Das hepatozelluläre Karzinom (HCC) ist weltweit der fünft häufigste maligne Tumor mit einer hohen Mortalitätsrate. Radiofrequenzablation (RFA) ist eine therapeutische Option für Patienten, die auf Grund des fortgeschrittenen Tumorstadiums nicht länger mittels Resektion oder Transplantation behandelt werden können. Das Ziel dieser Arbeit war es, ein Zellkulturmodell zu entwerfen, dass im Detail die Hitzeempfindlichkeit von humanen Zelllinien und die Wundheilung von gesundem Lebergewebe nach RFA im Schwein über einen längeren Zeitraum evaluiert.

Wir entwickelten ein Kokulturmodell um *in vitro* unterschiedliche Fibrosegrade zu simulieren und untersuchten diese hinsichtlich Hitzeempfindlichkeit und Überleben. Wir setzten diese Kokulturen, unterschiedlich lange (5-15 min), Temperaturen von 55 bis 100°C aus und ermittelten mit Hilfe von MTS Tests und Durchflusszytometrie die Anzahl der überlebenden Zellen. In unserem Großtiermodell behandelten wir 19 Schweine mittels RFA und euthanasierten sie nach unterschiedlicher Überlebensdauer (0d, 1d, 2d, 1w, 2w, 1m, 2m, 3m). Zur näheren Charakterisierung wurden Schnitte mittels CAB, MB, Caspase 3, Hsp70, Ki-67, Calprotectin, GFAP und α -SMA gefärbt um die Veränderungen nach RFA zu visualisieren.

Wir konnten zeigen, dass Fibrose einen großen Einfluss auf Form und Größe einer RFA Läsion hat. Um Tumorstadium oder -veränderungen zu vermeiden ist es wichtig in der Übergangszone der Läsion eine Temperatur von mindestens 75°C zu erreichen.

Hohe Temperaturen führen zu Nekrose und durch Hitze fixiertes Gewebe, welches scheinbar Reparatur und Abbau widersteht. Im Gegensatz dazu werden die apoptotisch sterbenden Zellen der Übergangszone von Fibroblasten ersetzt, welche im Laufe der Zeit eine Kapsel bilden; diese scheint als Grenze zu fungieren und eine chronische, lokale Entzündungsreaktion vergleichbar einer Fremdkörperreaktion zu bewirken.

Grundsätzlich können wir sagen, dass durch eine RFA eine akute Entzündungsreaktion ausgelöst wird. Die behandelte Stelle wird schnell durch eine Fibrosekapsel ersetzt, die in weiterer Folge eine Fremdkörperreaktion (chronische Entzündung) auslöst. Wenn das Tumorgewebe zur Gänze zerstört ist verschwindet die Läsion über Jahre ohne weitere Komplikationen.

1 Introduction

1.1 Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) accounts for 85% to 90% of primary liver cancer and is the fifth most common malignant tumour in humans [1]. A variety of risk factors are associated with HCC: hepatitis viruses (primarily B and C), vinyl chloride, tobacco, foodstuffs contaminated with aflatoxin B₁, heavy alcohol intake, non-alcoholic fatty liver disease, diabetes, obesity, diet, coffee oral contraceptives and hemochromatosis [2]. Hepatocarcinogenesis (fibrogenesis) is a complex, not fully understood process associated with accumulation of genetic and epigenetic changes that occur during development of the disease [2]. These genetic alterations are activation of cellular oncogens, inactivation of tumour suppressor, genomic instability, overexpression of growth and angiogenic factors and telomerase activation. The major clinical risk factor (70-90%) for HCC development is cirrhosis [3].

1.1.1 Liver fibrogenesis

Liver fibrosis is a reversible scarring response that occurs in almost all patients with chronic liver injury [4]. It could be caused among others by exposure to hepatitis viruses, vinyl chloride, tobacco, food contaminated with aflatoxin B₁, heavy alcohol intake, non-alcoholic fatty liver disease, diabetes, obesity, diet, coffee, oral contraceptives and hemochromatosis [2].

Fibrogenesis is a wound healing response of the liver in which damaged regions are encapsulated by extracellular matrix or scar caused by hepatic stellate cells (HSC; also known as Ito cells). They are quiescent cells in normal liver, whose main function is to store vitamin A and to maintain the normal basement membrane type matrix [5]. HSC are pericytes, that are located in the perisinusoidal space (between the sinusoids and hepatocytes) called the space of Disse [6]. Liver damage leads to apoptosis of hepatocytes and induces hepatic fibrosis (Figure 1). Kupffer cells, HSC and healthy hepatocytes internalize and degrade apoptotic bodies from dead hepatocytes, which lead to HSC activation [6, 7].

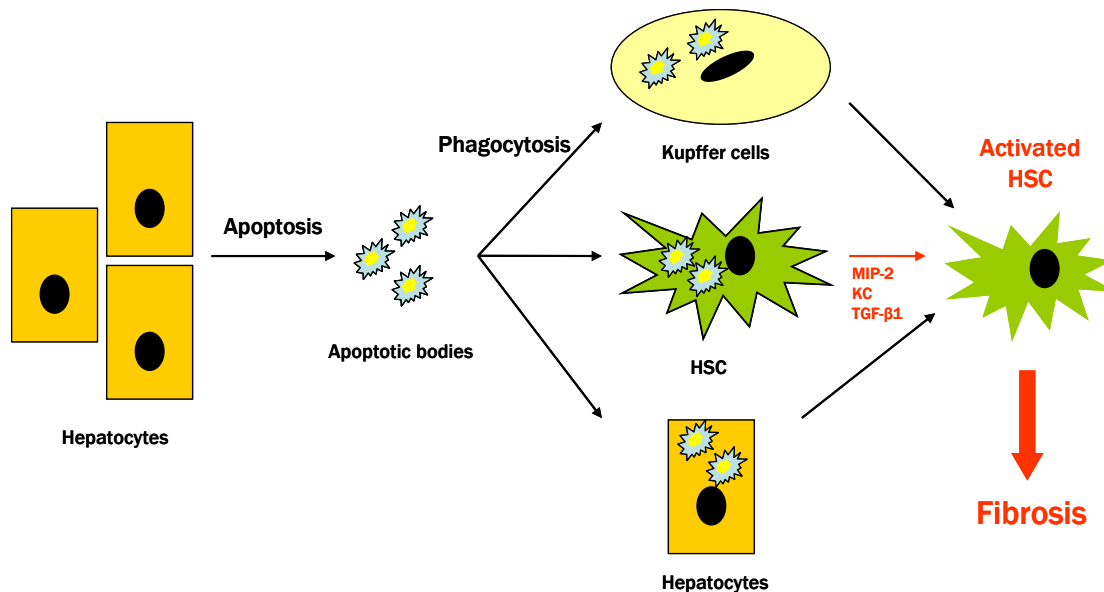


Figure 1: Hepatocyte apoptosis induces hepatic fibrosis.

In response to injury, apoptotic hepatocytes undergo DNA fragmentation and formation of apoptotic bodies, which are phagocytosed by Kupffer cells, hepatic stellate cells (HSC) or healthy hepatocytes. Kupffer cells and HSC release profibrogenic cytokines (TGF- β 1, MIP-2 and KC) triggering activation of HSC and collagen type I production [8].

The role of TGF- β 1

Transforming growth factor beta (TGF- β) consists of three isoforms in vertebrates: TGF- β 1, TGF- β 2 and TGF- β 3. TGF- β 1 is known as modulator of cell proliferation, cell differentiation and fibrogenesis [9, 10]. The induction of collagen expression by TGF- β is mediated by the phosphorylation of SMAD and SMAD3, and subsequent nuclear translocation of the SMAD complex [11]. It is expressed in normal and fibrotic liver, with an increase in cirrhosis and experimental hepatic fibrosis [9].

TGF- β 1 mediates the cross-talk between parenchymal, inflammatory and collagen expressing cells. It is released by activated Kupffer cells and sinusoidal endothelial cells to trigger apoptosis in hepatocytes [7]. Additionally, it also induces the activation and recruitment of inflammatory cells into injured liver and the differentiation of liver resident cells (fibroblasts, HSC, epithelial cells) into collagen producing myofibroblasts producing type I, III and IV collagen, proteoglycans and glycoproteins (Figure 2) [12, 13]. On the other hand, activated HSC themselves can secrete TGF- β 1, which leads to an autocrine loop [12]. Hepatocytes do not produce TGF- β 1 [14]. Furthermore, activated HSC secrete inflammatory cytokines (such as TGF- β , monocyte chemotactic

protein 1, RANTES, IL-10, IL-8) and up-regulate adhesions molecules (integrins, VCAM-1, ICAM-1), which cause lymphocyte recruitment and activation [12].

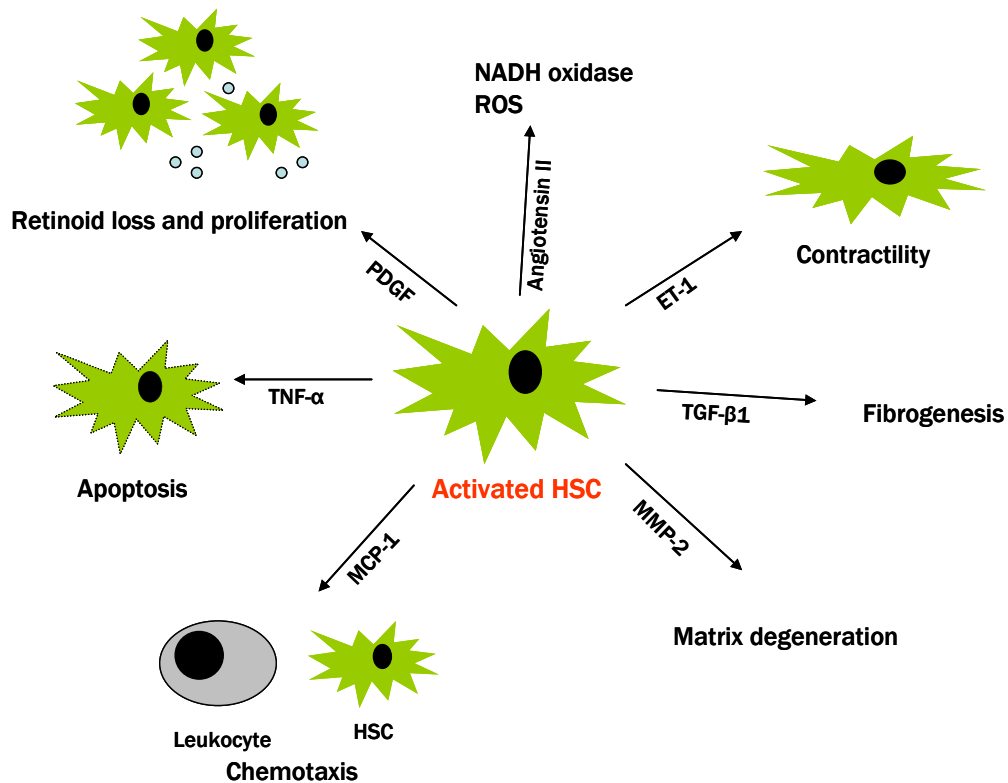


Figure 2: Characteristics of activated HSC.

In response to profibrogenic stimuli, activated hepatic stellate cells (HSC) lose lipid droplets and obtain myofibroblastic phenotype, contractile capability and differentiate into collagen type I-producing cells. Activated HSC induce lymphocyte recruitment and control matrix degradation. (PDGF, platelet-derived growth factor; ROS, reactive oxygen species; TGF- β 1, transforming growth factor- β 1; TNF- α , tumour necrosis factor- α) [8].

Fibrogenesis vs. regeneration

Chronic liver injury is associated with accumulation of matrix proteins, which leads over time to fibrosis. In parallel a continuous regeneration stimulus is induced leading to further alteration of the hepatic architecture and vascular structures; ultimately leading to cirrhosis, which is associated with nodule formation and organ contraction [4, 15, 16]. On the other hand it was found that fibrosis can undergo spontaneous resolution after successfully treatment of the eliciting disease [14]. It was documented by alcohol-induced liver injury, chronic hepatitis B, C and D, hemochromatosis, secondary biliary cirrhosis, non-alcoholic fatty liver disease and autoimmune hepatitis. The time duration of significant regression is dependent on the liver disease and its

severity [14]. There are several studies in rats, which mention that ECM overproducing HSC become apoptotic and lead to liver recovery. But the question “does liver fibrosis reach a point where it becomes irreversible?” is so far not answered. It can only be suggested that this happen due to a combination of factors [17].

1.1.2 Diagnosis and therapy

Early diagnosis in HCC patients is crucial in order to enrol effected patients in proper therapy regimens. HCC is identified and characterized on the basis of a liver biopsy, which is an invasive procedure and can cause complications [2, 18, 19]. Following serological liver parameters are used for characterization and staging of liver diseases (so-called “liver function testes”): bilirubin, alanine transaminases, aspartate transaminase, alkaline phosphatase and gamma-glutamyltranspeptidase [20]. For HCC diagnostics, in literature a lot of potential HCC markers, which are compiled in Table 1 [21], are under investigation. These most promising markers for detection of HCC are Alpha-fetoprotein (AFP), Lens culinaris agglutinin reactive AFP (APF-L3), Des- γ -carboxy prothrombin, α -L-fucosidase, Glypican-3, P-aPKC- ι , E-cadherin, β -catenin, human carbonyl reductase, squamous cell carcinoma antigen, serum proteomics, Golgi protein 73, Chromogranin A, vascular endothelial growth factor, hepatocyte growth factor, TGF- β , serum anti-p53 and nervous growth factor. Among these APF is the most promising marker and in combination with hepatic ultrasonography (US) already used to detect HCC in cirrhotic patients [21]. The increase of AFP levels $>500\text{ng/ml}$ is correlated with the tumour size. However, about 80% of small HCCs show no increase the of AFP level [22]. AFP-L3 is an isoform of APF and differs due to lectin lens culinaris agglutinin binding capability from the others. This protein can be detected in about 35% of all small HCC patients ($<3\text{cm}$) [23]. P-aPKC- ι , E-cadherin and β -catenin play an important role in tight-junction formation among tumours cells [21]. Glypican-3 interacts with several growth factors and regulates their activity. It also acts as tumour suppressor. It is used to differ between malignant and benign hepatic lesions, because it is undetectably in healthy tissue and benign tumours. Overexpression is a sign for malignancy [24]. Other potential markers are listed in the table below (Table 1)

Table 1: Overview of potential serum marker for HCC [21]

HCC marker	Principal use
Alpha-fetoprotein (AFP)	HCC early diagnosis, monitoring, and recurrence
Lens culinaris agglutinin reactive AFP	HCC early diagnosis and prognosis (vascular invasion and intrahepatic metastasis)
Des- γ -carboxy prothrombin	HCC early diagnosis and prognosis (early portal vein invasion and metastasis)
a-l-fucosidase	HCC early diagnosis
Glypican-3	HCC early diagnosis
P-aPKC- ι , E-cadherin, b-catenin	HCC prognosis
Human carbonyl reductase	HCC prognosis
Squamous cell carcinoma antigen	HCC early diagnosis
Serum proteomics	HCC early diagnosis
Golgi protein 73	HCC early diagnosis
Chromogranin A	HCC prognosis and possible therapeutic treatment
Vascular endothelial growth factor	HCC prognosis (metastasis development)
Hepatocyte growth factor	HCC prognosis and disease recurrence
Transforming growth factor- β	HCC progression
Serum anti-p53	HCC prognosis (poor differentiation)
Nervous growth factor	HCC prognosis and progression

Additionally hepatic fibrosis scans via computer tomography (CT) or US could be used for staging and grading of fibrosis as well as cirrhosis [25, 26]. *Colli et al.* compared in a systematic review the accuracy of US, spiral CT and magnetic resonance imaging (MRI) to the pathology of explanted livers [27]. Their results support the use of US as a specific diagnostic tool at least in patients with underlying cirrhosis and a high probability of HCC. However, they mentioned that US has a poor sensitivity and is not adequate for screening purposes. The role of CT and MRI remains to be defined, particularly as additional tests aimed at confirming positive US results [27]. Up to now, none of these tests can replace liver biopsy as the golden standard [28].

When a tumour develops in a cirrhotic liver, therapy depends on tumour size, localization as well as staging and grading (see 1.3). Patients with HCCs fulfilling the Mazzaferro criteria [29] are treated by liver transplantation (LT), but this treatment approach is limited by the lack of human donor organs [30]. Another possibility to potentially cure HCC is surgical resection [31]. In many cases, resection or transplantation is not feasible any more due to the advanced stage of the HCC and the underlying liver cirrhosis at the time of diagnosis. Radiofrequency ablation (RFA) is a therapeutic option for these patients as well as selected transcatheter arterial chemoembolization (TACE), percutaneous ethanol injection therapy (PEIT) or percutaneous microwave coagulation therapy (PMCT) [32-34].

1.2 Other types of liver neoplasm and metastases

About 60-80% of patients with colorectal carcinoma, pancreatic carcinoma, breast cancer, neuroendocrine tumours, and other tumour types will develop liver metastases. Surgical resection is the golden standard for treatment, but about 75% of these malignancies are unresectable. In these cases hepatic arterial infusion chemotherapy (HAC), TACE, RFA or radioembolization (RE) could be chosen [35-37]. Below the most frequent hepatic metastases are specified.

1.2.1 Colorectal metastases

About 50-80% of patients with colon carcinoma will develop liver metastases and result in jaundice, abdominal pain and ascites [38, 39]. A possible predilection for metastases arises because of the portal venous drainage from the colonic or rectal primary tumour to the liver. An average survival of untreated patients from the time of diagnosis is about 6 to 9 months [38, 39]. The only curative treatment for liver metastases is surgical resection, which is technically feasible in about 20% of all patients [39]. Other considered treatments are cryotherapy, RFA, PEIT, laser-induced thermotherapy (LITT), chemotherapy or portal vein embolization, which are often used to increase the resectability of the metastases [38-40]. Additionally colorectal carcinomas are usually sensitive for chemotherapeutic agents as fluorinated pyrimidine 5-fluorouracil (5-FU), Tumudex (ZD 1694), Irinotecan (CPT11) or Oxaliplatinum. Often these agents are only used for a live prolonging palliative therapy [38, 39].

1.2.2 Uveal melanoma metastases

Primary tumours arise in the uveal tract, which implicates the iris, ciliary body and the choroid. The liver is the exclusive site of systematic metastasis in about 40% of the patients [41]. About 90% of metastases are in the liver, the other 10% remain in lung, skin, bone and brain [42]. After diagnosis the treatment options are limited and the life expectancy is poor (median survival time between 2,2 and 12,5 months). Therapeutic options are partial hepatectomy, and if possible followed by HAC [41, 42].

1.2.3 Breast metastases

More than 50% of patients with breast cancer develop liver metastases, which is attended with poor prognosis [43, 44]. The liver is the primary metastatic site in 12-

15% of patients with metastatic breast cancer [45]. Depending on the literature the median survival time ranges from 1 to 12 months [43, 44]. The best therapy for patients with solitary liver metastases is surgical resection with or without chemotherapy, but most patients are not suitable for surgery. These patients are treated with chemotherapy or endocrine therapy. In recent years RFA and TACE have become alternative treatments for high surgical risk patients [35, 43].

1.2.4 Neuroendocrine metastases

Gastrointestinal carcinoids and pancreatic islet cell tumours frequently metastasize to regional lymph nodes, the bones, and the liver. Neuroendocrine metastases can cause liver dysfunction and result in a variety of hormonal syndromes (*i.e.* carcinoid syndrome) due to their ability to synthesize and secrete hormonally active polypeptide products (e.g. octreotide). Hepatic resection, ablation and TACE have been shown to be very effective for treatment of these metastases. [35, 46].

1.2.5 Cholangiocarcinoma

Cholangiocarcinoma (CCA) is a cancer of the biliary epithelium and can develop along the biliary tree. It could be classified as intrahepatic (in the liver), hilar (at the confluence of right and left hepatic ducts) and extrahepatic (distal the liver) [47, 48]. More than 90% of the bile duct cancers are well-differentiated and mucin-producing adenocarcinomas. Other types are squamous cell carcinoma, small cell carcinoma and sarcoma [47].

Most frequently known risk factors for CCA include primary sclerosing cholangitis, intrahepatic biliary stones, congenital liver malformations (*i.e.* Caroli's disease, congenital fibropolycystic disease), Hepatitis B and C, infections with parasitic liver flukes (*Opisthorchis viverrini* or *Clonorchis sinensis*), toxins (including alcohol), cirrhosis, diabetes and obesity [47, 48]. Analogue to other liver malignancies hepatic resection is the golden standard therapy [49], but survival is extremely poor (an average 5-year survival of 5%). Sometimes photodynamic therapy is used for patients with locally advanced disease [50]. Furthermore, CCA is highly resistant to chemotherapy, but systemic chemotherapy may be considered in patients with advanced CCA [50]. According to recent studies gemcitabine and cisplatin are very promising for standard therapy for locally advanced and metastatic CCA [51].

1.3 Therapy options for HCC

1.3.1 Surgical resection and liver transplantation

In 90% of HCC patients cirrhosis is present, which is associated with higher intra- und perioperative morbidity and mortality rates. The postoperative mortalities are less than 3% for non-cirrhotic patients and range from 7 to 25% for cirrhotic patients [52]. It is discussed controversial if long-term survival is also influenced by cirrhosis. Other factors influencing the outcome after resection are the tumour multiplicity as well as tumour size [52]. It was reported that patients with multiple tumours have a higher recurrence rate and a lower survival rate [53]. Often large tumours can't be resected due to deficient residual liver function. If the remaining liver tissue is not sufficient patients would develop liver failure [54]. Zhou reported a 1-, 3-, 5- 10- year recurrence rate of 9,2%, 38,8, 54,9 and 85,0% after hepatic resection. This tumour recurrence could be caused by three different occasions. 1) The first tumour was not completely resected. 2) An unrecognized multifocal primary tumour caused the recurrences. 3) A new primary tumour developed [55].

Patients with large (greater than 10cm [56]) or non-resectable HCC are the most frequent candidates for LT [57]. Total liver resection (hepatectomy) offers the chance to cure HCC and the underlying cirrhosis at the same time [56]. However, an increased organ shortage followed from long time on LT waiting list led to development of alternative, bridging methods. To identify patients with a maximal benefit from LT different criteria and scores were created (MELD score [58], Milan and Mazzaferro criteria [29, 59]). The prognosis after LT depends on the status of the recipient, donor organ and technical complications during operation. The postoperative complications can be divided into medical and surgical [60]. Surgical complications are among others vascular, biliary or other complications as *e.g.* evisceration, wound infection, incisional hernia or pleural effusion. Possible non-surgical complications are cholangitis, non-surgical bleeding or infection [60]. Immunosuppression is essential after LT. However, it could be shown that tumour recurrences were increased by immunosuppressive medication, because they inhibit the body's own tumour surveillance. In experimental (*in vitro* and animal) studies it was shown that calcineurin-inhibitors, tacrolimus and cyclosporine enhance the cellular growth of malignant HCC cells [61]. On the other hand it was shown that rapamycin (also called sirolimus) inhibits tumour growths in

cells lines and inhibits primary and metastatic tumour growths *in vivo* [62]. So far no data from prospective randomized controlled studies are available [63].

1.3.2 Transcatheter arterial chemoembolization

By interrupting the arterial supply, chemoembolization deprives the tumour of its major nutrient source, which results in ischemic tumour necrosis [64]. TACE has been proposed as a treatment for patients with unresectable hepatocellular carcinoma to bridge the waiting time between the diagnosis and LT. When combined with other procedures such as PEIT, TACE has been more successful at achieving survival rates and is equal to those obtained after surgical resection in similar patient populations [65].

1.3.3 Percutaneous ethanol injection therapy

Ethanol has the ability to penetrate the tumour cells, producing coagulative necrosis as a consequence of dehydration of cytoplasmic proteins. Additionally, it causes the thrombosis of tumoural microcirculation, which results in necrosis of endothelial cells and platelet aggregation, followed by ischaemia of the neoplastic tissue [66]. PEIT has proven to be highly effective in single HCC up to 3 cm, obtaining a complete response in 80% of cases, being well tolerated and with a limited risk of minor complication [66]. In larger and/or multinodular HCC the efficacy is reduced to 50% of complete response in nodules between 3 and 5 cm [67] and to a lower rate in larger tumours. The size of the tumour also influences the rate of local recurrence. [66, 68, 69]. Furthermore, PEIT maintains the advantage of allowing treatment of tumours near sensitive organs and tissues, and avoids the problem of the “heat-sink” effect adjacent to vessels [68].

1.3.4 Percutaneous microwave coagulation therapy

Microwave ablation induces tumour destruction by using devices with frequencies of at least 900 MHz achieving constant temperatures of at least 60°C in target tissues [70-72]. This treatment can be applied to HCC, intrahepatic metastasis, and other lesions with an impaired liver function. Compared with other minimally invasive treatments such as PEIT or TACE, PMCT has been evaluated to offer a potentially complete tumour ablation and to also be clinically equivalent to a hepatic resection [67, 73, 74].

1.3.5 Laser-induced thermotherapy

(LITT has recently applied for a minimally invasive technique in local treatment of liver metastases. A low power laser applicator, which delivers light energy through thin optical fibres, leads to a well-defined area of coagulative necrosis. This causes destruction of the tumour by directly applied thermal energy and limits the risk of damage for surrounding structures, such as nerves or vessels. [75-78].

1.3.6 Radiofrequency ablation

RFA is used as treatment for lung, liver, kidney and bone tumours. In contrast to resection and transplantation, RFA is a minimally invasive treatment and can be performed percutaneously in HCC patients. It is therapy of choice for patients with large, unresectable tumours (due to poor liver function, multifocal disease) or tumours in complicated anatomical locations. [79]. To be suitable for RFA patients should have one HCC nodule less than 5 cm or as many as three nodules less than 3 cm in maximum diameter. Additionally, the underlying liver disease should be no more advanced than Child Pugh class B [80]. RFA is not only used for curing HCC; but also for bridging till liver transplantation. In this case RFA is used to slow down the tumour progression and decreases so the waiting list dropout rate. It prolongs not only patients' life but also decrease tumour recurrence after LT [81].

Lethal complications after RFA are intra-abdominal bleeding, liver failure, cardiac complications, and bile duct stricture –associated complications [79, 82]. Due to the simplicity, low-cost and the low morbidity rates the technology evolved quickly and shortly found its way as tool of surgeons and radiologists. However at the same time several concerns raised [83]. Differences in hepatic and tumour perfusion cause variations in size and shape of the lesions and can hardly be calculated (see 1.3.6.1). This could be linked to high local recurrence rates. [84-86]. Secondary, possible promotion of intrahepatic growth of remaining tumour cells, because of (not yet proven) immunological and biological effects of heat trauma [83, 87-89] have to be taken in consideration. Therefore the risk factors include subcapsular tumour localization, a high degree of poor differentiation and a high AFP baseline [86]. Obara *et al.* showed in a cell culture study investigating the heat sensitivity of three different HCC cell lines that insufficient RFA, which does not kill all tumour cells, may induce further malignant transformation of HCCs [90]

RFA uses electromagnetic energy sources to generate heat. Therefore, electrode probes are placed into a tumour percutaneously during open or laparoscopic surgery and the tissue around this electrode is coagulated by a radiofrequency generator [81]. High-frequency (375-500 kHz) electromagnetic waves displace molecules within the tissue in alternating directions. Changes in the energy state of molecules result in an ionic agitation and localized increase in temperature [91, 92]. This ionic movement causes heating of the tissue and leads to a necrotic coagulation of the tumour cells [93].

Temperatures between 46°C and 60°C are associated with irreversible cellular damage that is proportional to the exposure time [91, 94]. Between 60°C and 100°C a nearly instantaneous indication of protein coagulation occurs with irreversible damage to mitochondrial and cytosolic enzymes of the cells [91]. Temperatures above 105°C result in tissue boiling, vaporization and carbonization. Therefore the key aim for thermal therapy is achieving and maintaining a 60-100°C temperature range throughout the entire target volume [66, 91] in order to solely destroy the affected, but not the healthy liver.

The progression of tissue injury after RFA may represent two distinct phenomena. At first (**initial phase**) cells that are lethally damaged with heat application may initially seem morphologically normal, with the full extent of injury only becoming evident well after the end of the initial heat stimulus. And secondary, there will be an increase in tissue injury caused by progressive death of cells that were still viable after heating (**progressive phase**) [95].

1.3.6.1 Factors influencing RFA outcome

Heat sink effect

This technique is already used routinely in human livers, but so far no experimentally validated model exists to be able to accurately plan the procedure. However, several patient specific factors determine the exact heating up and heat transportation process in the hepatic tissue. Among others, blood flow, blood pressure [25, 96, 97] as well as the composition of the liver tissue (amount of fibrosis) [98] seem to have a crucial impact on RFA outcome. Blood flow leads to the so-called “heat sink” effect during RFA through heat transportation and cooling [19, 31, 87, 99] and therefore limits the necrotizing effect, which results in a reduced coagulation size. To show a direct correlation between blood flow and RFA ablation sizes the Pringle manoeuvre is used. During Pringle manoeuvre the hepatic artery and the portal vein is clamped [25, 96], or

balloon catheters, placed in the main trunk of the portal vein, are inserted to reduce the blood flow in order to document its influence on RFA success is used. Yoshimoto *et al* revealed that a decrease in portal flow volume of 50% led to an increase of the ablation size at the same time [25]. Follow-up scans after RFA are mandatory during the following months to verify success of the intervention.

Oven effect

The cirrhotic tissue around the tumour may influence the outcome of the RFA by behaving as a thermal insulator. The lower thermal conductivity of the background tissue leads to a lower heat transmission from the inner tumour compartment to the surrounding outer compartment. The oven effect may also explain the differences in results between tumour types and why the necrotic area became larger as calculated [98, 100].

1.3.6.2 Changes in liver lesion after RFA

In most hospitals patients that were treated with RFA routinely have follow up scans (CT or MRI) after a few months. So far there exist not many data about the development of the coagulation necrosis after RFA. Kuehn *et al.* found that the necrosis zone is larger 48 h after LITT then immediately after, but they can't exclude further increase because they investigated only 48 hours after RFA. After 3 months (CT scan) the necrosis area was significantly decreased [101]. At CT evaluation a few days after RFA a hyperattenuating rim around the destruction site is often visible on CT scan, reflecting post interventional hyperemia or tissue regeneration [79, 102, 103].

The RFA lesion can be classified in four different zones including the application zone, the central zone, the transition zone as well as reference zone (Figure 3). In the **application zone** the tissue is directly exposed to the thermal electrode and characterised by irreversible damage (carbonisation). The **central zone** is adjacent to the application zone and consists of necrotic tissue with damaged structures (main part of the lesion). The **transition zone** surrounds the central zone and is morphologically described as hemorrhagic area, containing apparently undamaged liver cells with signs of tissue infiltration with blood cells. In this zone cells are eosinophilic with condensed chromatin; however intracellular connections are maintained. The **reference zone** contains the normal, undamaged tissue surrounding the lesion [77, 91, 104].

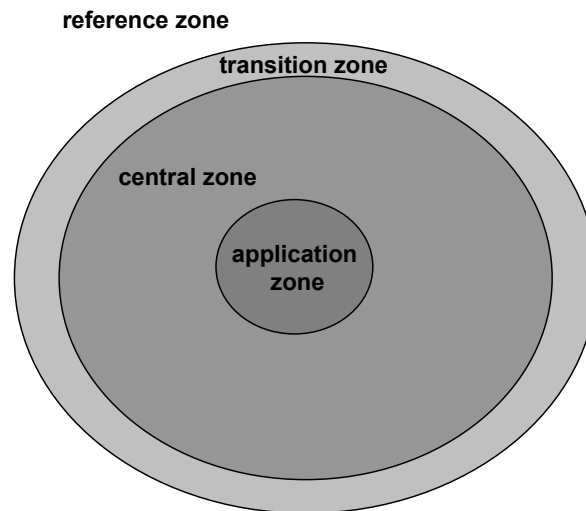


Figure 3: Different zones of a RFA lesion

Rai *et al.* reported minimal apoptotic activity in the transition zone 30 min after experimental RFA in porcine liver [105]. Ohno *et al.* found the peak of Caspase 3 activity 2 hours after microwave coagulation therapy in liver tissue homogenates [106]. Vanagas *et al.* determined that apoptosis leads to further cell death, and is activated in the majority of cells at a distance of more than 9-10 mm for the rim of necrosis zone [104].

1.4 Wound healing in healthy tissue

Wound healing after injury in healthy tissue occurs in three overlap phases: inflammation, proliferation and remodelling. As early reaction to the wounding the clinically recognizable cardinal signs of inflammation occur: *rubor* (redness), *calor* (warmth), *tumour* (swelling), *dolour* (pain), and *functio laesa* (loss of function) [10, 107]. Before the inflammation phase is initiated, the clotting cascade is responsible for haemostasis. After wounding, blood comes in contact with collagen and triggers blood platelets to secrete inflammatory factors (serotonin, bradykinin, prostaglandin, prostacyclines, thromboxane and histamine [108]). To control bleeding these platelets also express glycoproteins to form aggregates [109]. Furthermore, they release platelet derived growth factor (PDGF) and transforming growth factor beta (TGF- β) to initiate the repair process [10, 107]. These factors initiate the chemotaxis of neutrophils, macrophages, smooth muscle cells and fibroblasts [110].

1.4.1 The inflammatory phase

White blood cells are the predominant cells for the first 3 days after wounding with their peak at approximately 48 h. Neutrophils arrive within an hour at the wound site and attach to endothelial cells in the vessel walls surrounding the wound. Their major function is to remove foreign material, bacteria and non-functional host cells and damaged matrix components [10, 110]. Shortly after circulating monocytes enter the wound and mature into tissue macrophages [107], which are responsible for clearing the wound of all matrix and cell debris including fibrin and spent neutrophils [111]. TGF- β stimulates them to secrete additional cytokines including PDGF, basal fibroblast growth factor (bFGF), tumour necrosis factor alpha and IL-1 [10, 110]. Another leukocyte, the helper T cell, secretes IL-2, which promotes further T cell proliferation to augment the immunogenic response to injury [107].

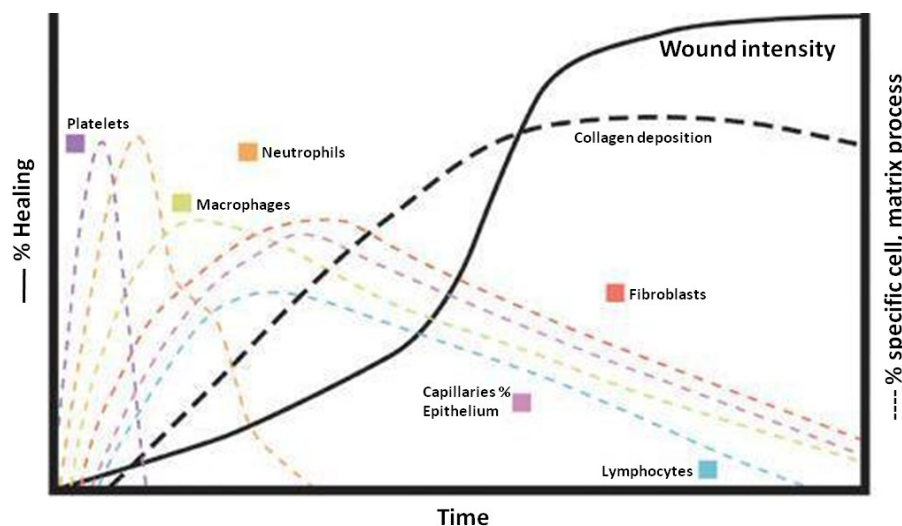


Figure 4: Presence of the different cells during wound healing process.

Figure modified after de la Torre *et al* [107].

1.4.2 The proliferative phase

Two to three days after injury, fibroblasts migrate to the wound and become the dominant cell type, peaking after 1-2 weeks. During the first week, fibroblasts start to produce glycosaminoglycans and proteoglycans, which are the ground substance for granulation tissue. Additionally they produce collagen in response to macrophage-synthesized bFGF and TGF- β [10, 107]. In normal tissue collagen provides strength, integrity and structure. When tissues are disrupted, collagen is needed to repair the

defect and restore anatomic structure and function. If too much collagen is deposited in the wound site, normal anatomical structure is lost, function is impaired and fibrosis occurs [110].

Furthermore, neovascularisation occurs concurrently with fibroblast proliferation when endothelial cells migrate to the wound site. It is stimulated by vascular endothelial growth factor, bFGF and TGF- β , which are released by epithelial cells, fibroblasts, macrophages, and vascular endothelial cells [10, 112].

Formation of granulation tissue in an open wound allows reepithelialisation, which means that epithelial cells migrate across the new tissue to form a barrier between the wound and the environment until the defect is covered. [110]. At this point, transformation of fibroblasts into myofibroblasts is induced by contact inhibition [107]. The process of epithelialisation is stimulated by the presence of epithelial growth factor (EGF) and TGF- α that are produced by activated wound macrophages, platelets and keratinocytes [110].

1.4.3 The maturation phase

For the first 6 weeks, new collagen production dominates the wound healing process and is deposit randomly in acute wound granulation tissue. Small capillaries connect to larger blood vessels and the water content of the wound decreases. The cell density and overall metabolic activity of the wound declines as well [10]. As the wound matures, collagen is remodelled into a more organized structure with increased tensile strength. Gradually, type I collagen replaces type III until the normal skin ratio of 4:1 is achieved [107, 108, 113]. As remodelling continues, matrix metalloproteinase collagenolysis achieves a steady state with collagen synthesis [114]. Tensile strength plateaus at 80% of the original strength approximately 1 year post injury [107].

1.5 Aim

This study was part of the EU 7 Framework Project “IMPPACT” (no. 2238779), which aimed to develop an intervention planning and monitoring application for RFA of malignant liver tumours. So far the outcome of the treatment is hard to predict. Heating is strongly influenced by perfusion of the liver (blood flow) and the condition of the organ (healthy, fibrosis, cirrhosis or HCC). It is indispensable to reach lethal temperatures during RFA, because it must be ensured that all tumour cells are destroyed to cure patients and preclude recurrence.

In this study we wanted to gain more information about the physiological parameters and picture the outcome of the RFA over three months by means of animal and cell culture experiments. These results are the basis for creating a bio-heat model, which is essential for modelling the RFA process in the liver. Based on this output a patient specific tool for the RFA should be created which will allow accurate planning and monitoring of the process in clinical applications.

In order to obtain the essential information for the planning tool, cell culture experiments were necessary to investigate the heat sensitivity of different liver cell lines, the influence of the liver composition on the cell survival (*in vitro* simulation of fibrosis) as well as the type of cell death after hyperthermia (apoptosis or necrosis).

In the course of the cell culture experiments, primary endpoints were the monitoring of cell viability dependent on exposure to heat over certain time spans as well as the impact of the grade of fibrosis/cirrhosis on the heat resistance in co-culture experiments. Secondary endpoints was distinguishing between necrosis and apoptosis in cell culture experiments dependent not only on the temperature the cells were exposed and the time of heat exposure but also on the composition of the cell cultures. Moreover, the effects of co-culture experiments as well as transwell experiments on cell survival after heat exposure were evaluated.

In order to simulate human conditions, the pig was chosen for the animal experiments, due to its size and liver composition. The animal experiments were performed in order to gain information on the fibrogenesis of the capsule surrounding the RFA zone developing after ablation, on the inflammatory response after RFA in healthy livers,, in

order to evaluate The heat stress and apoptosis in the transition zone and surrounding tissue as well as the influence of blood flow on RFA outcome.

Primary endpoints in the animal experiments were establishing the RFA procedure in an animal model as well as the induction of fibrosis/cirrhosis in the pig. Moreover, the changes in the RFA zone were evaluated over time. Secondary endpoints were the evaluation of heat shock proteins as well as the occurrence of apoptosis in the liver tissue in healthy pig liver tissue as well as in cirrhotic pigs over time. Moreover, changes in CT scans were documented and compared to histological and immunohistochemical results. The animal experiments aimed to gain sufficient information delivered to the partners of the EU project in order to optimize human procedures in future.

2 Material & Methods

2.1 Co-culture experiments with MRC-5 and HepG2

The human liver hepatocellular carcinoma cell line HepG2 (ATCC# HB-8062, Rockville, MD, USA) and the human lung fibroblast cell line MRC-5 (ATCC# CCL-171, Rockville, MD, USA) were cultivated at 37°C in MEM containing additionally 10% FBS and 1% P/S in a humidified atmosphere (5% CO₂). Cells (3×10^4 cells/100µl) were seeded in cell culture treated 96 well plates and cultivated overnight. For the experiments, the culture medium was replaced by preheated medium. Then the plates were incubated for different time spans (5, 10 or 15 min) in a heating cabinet preheated at different temperatures according to the experimental setup (55°C, 65°C, 75°C, 85°C and 100° C respectively). Thereafter, the medium was replaced by medium at 37°C. Metabolic activity was tested either immediately after exposure to the different temperatures, after 24 h as well as after 48 h of cell culture in culture medium at 37°C as mentioned below. In order to simulate different grades of fibrosis, co-cultures of HepG2 and MRC-5 cells were cultivated and treated the same way as the single cultures at 37°C prior to exposure to the different temperatures as mentioned above. 25%Hep/75%MRC means a co-culture of 25% HepG2 and 75% MRC-5 cells in one well and 50%Hep/50%MRC terms a co-culture, which consists in equal parts of HepG2 and MRC-5 cells.

2.1.1 Metabolic activity testing

For testing the metabolic activity of the cells MTS-test (CellTiter 96® AQueous One Solution Cell Proliferation Assay) was used exactly as described by the manufacturer. After the heating experiments, the hot medium was replaced by 100µl medium at 37°C and spiked with 20µl MTS reagent. The absorbance was measured 2 hours after incubation with the MTS reagent at 37°C at wavelengths of 490nm and 650nm using a SpectraMax Plus 384 (Molecular Devices, USA). The data were determined by calculating the differences of the two wavelengths. Furthermore we measured the metabolic activity 24 and 48 h after heating using the MTS-test.

2.1.2 Viability staining

500µl medium containing 2×10^5 cells (HepG2 and MRC-5) were seeded in 24 well plates and cultivated overnight. After 24 h of culture, the cells were exposed to different temperatures as described above. Then after withdrawing the supernatant, 150µl Fluoresceindiacetate (FDA) (5mg FDA dissolved in 1ml acetone and 2,5ml cell culture Medium) and 150µl Propidium Iodide (PI) (1mg PI dissolved in 1ml distilled water) were added to 150µl remaining medium. The FDA dye passed through the membrane and was cleaved in viable cells that led to a green fluorescence [97]. For FDA a blue filter (wiba) was used with wavelengths from 460 to 490nm. PI binds specifically to RNA and DNA mostly by intercalation of double strands [96] green filter (wig) was used for visualization of PI (red) with wavelengths from 520 to 550nm. Green cells were defined as viable and red ones as non-viable (Figure 17). The pictures were taken with an inverted Olympus IX51 microscope and Cell[^]D Software (Olympus, Germany).

2.2 Co-culture experiments with LX-1 and HepG2

In this subsequent experiment the hepatic stellate cell line LX-1 (gift from Prof. Friedman) was used parallel to MRC-5 in the co-culture experiments. LX-1 were cultivated in DMEM containing additionally 10% FBS and 1% P/S, 1% L-glutamine in a humified atmosphere (5% CO₂). Cells (3×10^4 cells/ 100µl) were seeded in cell culture treated 96 well plates and cultivated overnight. Additionally all cells (100% LX-1, 100% HepG2 and their co-cultures) were pre-incubated in MEM and 2,5ng/ml recombinant human tumor growth factor-beta 1 (TGF-β1; Biovision, Mountain View, CA, USA) (5µg solved in 100µl 1X PBS (50µg/ml); aliquoted as 500ng/ml stock) 18 hs before heating (55°C for 5, 10, 15 min, 65°C for 5, 10, 15 min, 75 for 5, 10 min and 85°C for 5 min).

2.2.1 Metabolic activity testing

For measurement of the metabolic activity the MTS-test was performed as described in 2.1.1

2.2.2 Viability staining

500µl medium containing 2×10^5 cells (HepG2 and LX-1) were seeded in 24 well plates and cultivated overnight. Thereafter the cells were exposed to different temperatures as described above. The Live/Dead® Viability/Cytotoxicity Kit was used according to manufacturer's protocol. 15 min after heating the cells were incubated for another 15 min at 37°C with 200µl of the staining solution (containing 1µl of Calcein AM and 4µl EthD-1 in 2ml 1X PBS). Photodocumentation was performed with an inverted Olympus IX51 microscope and Cell[^]D Software. Alive cells emerge in green fluorescence and dead cells are stained red.

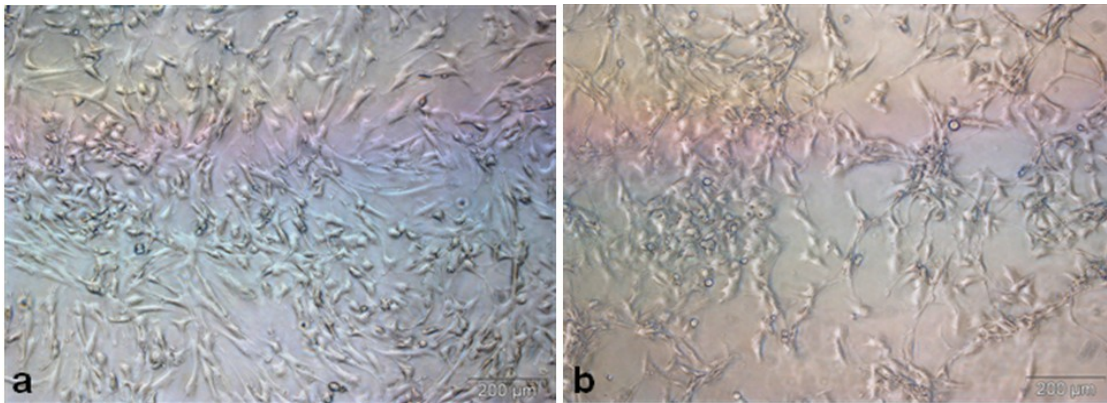


Figure 5: Co-Culture of LX-1 and MRC-5 with HepG2.

Images show co-culture of the hepatic stellate cells (LX-1) and human hepatocellular carcinoma cell line (HepG2) (a) and the human lung fibroblast cell line (MRC-5) and HepG2 (b) after 24 h of cell culture.

2.3 Transwell experiment

Different numbers of LX-1 cells were seeded in 12 well plates (Corning, NY, USA) and treated with 2.5 ng/ml TGF-β1 for 18 h (Figure 8a) according to literature [115-117].

Additionally HepG2 cells were seeded onto a transwell insert (Corning, NY, USA) with a lower compartment containing only culture medium (Figure 8b). 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%) (Figure 6 and Figure 7). For heating and cultivating afterwards the compartments were combined to gain 100% of cells (*e.g.* 75% HepG2 and 25% LX-1 cells; Figure 8C).

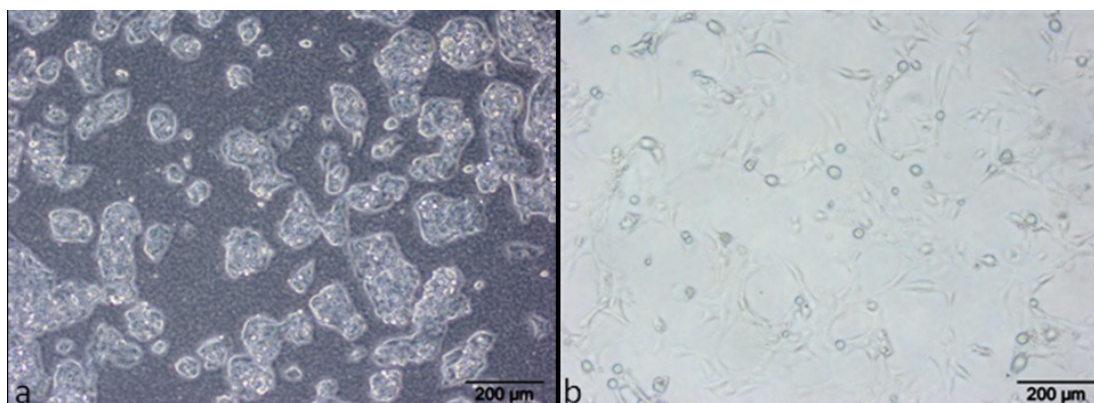


Figure 6: Cultivating HepG2 and LX-1 cells in transwells.

HepG2 cells (a) are cultivated on the membrane compartment separated from LX-1 cells (b). The different grades of fibrosis: 1×10^5 (100%), $7,5 \times 10^4$ (75%), 5×10^4 (50%) and $2,5 \times 10^4$ (25%).

Immediately before heating the supernatant was withdrawn and the inserts (containing HepG2 cells) were put into the wells (containing LX-1 cells) to get the desired amount of fibrosis (Figure 8c). Then, 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 the heating cabinet. After heating, the warm medium was exchanged to 37°C growth medium. One part of the plates was immediately used for metabolic activity testing; the other part was cultured over 24 and 48 h (LX-1 and HepG2 cells together in one well). Then, analysis of metabolic activity was performed as described below.

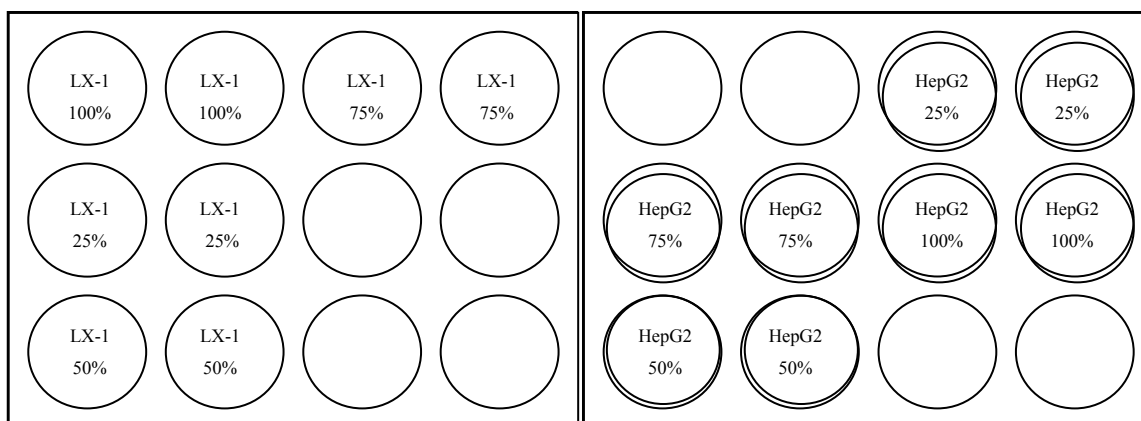


Figure 7: Cells prepared for transwell experiment:

The cells were seeded in different compartments and cell amounts to simulate

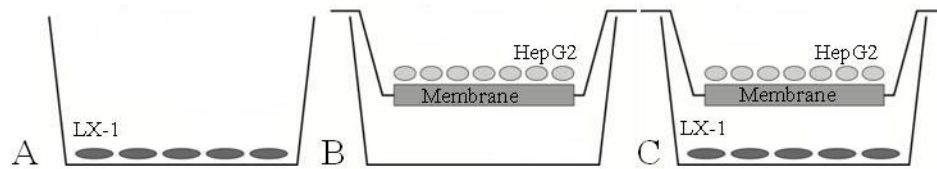


Figure 8: Cells prepared for experiments:

LX-1 cells were seeded onto the lower compartment and incubated with TGF- β 1 for 18 hours overnight (a); HepG2 cells grow separately on the membrane of the upper compartment (b). For the heating experiment and the follow-up culture the cells stay together in one well (c).

2.3.1 Metabolic activity testing

For MTS test cells were separated the compartments again and incubated them separately with 500 μ l Medium and MTS reagent (1:5) over 4 hours (Promega, Austria). Thereafter 2x 120 μ l were transferred into a 96 well plate and measured via SpectraMax 384 (Figure 9:).

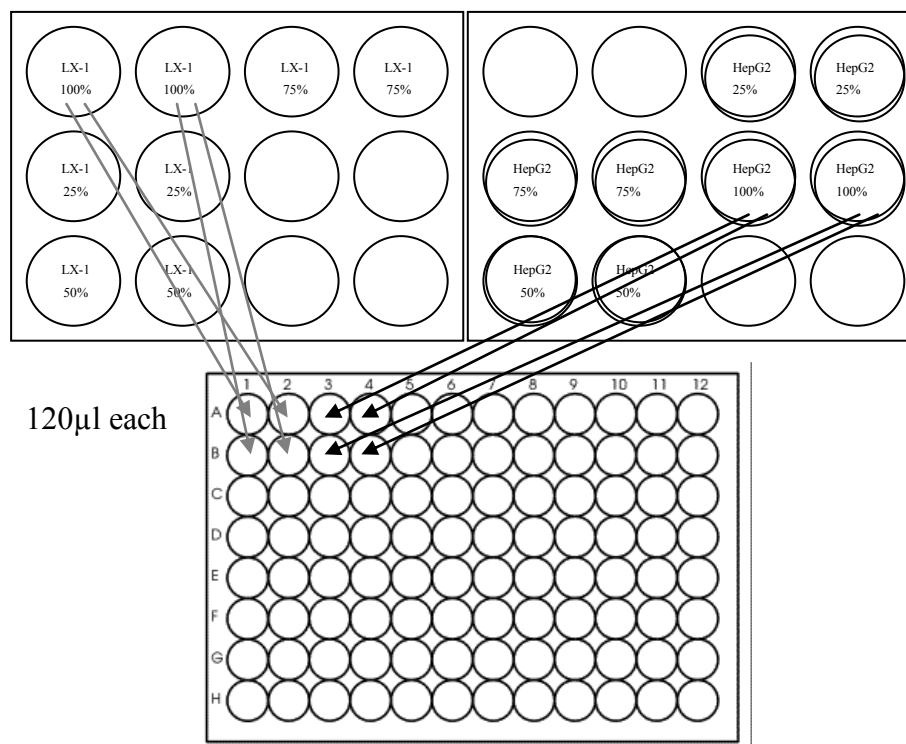


Figure 9: Measurement of cell metabolism via MTS test.

After heating the warm medium was exchanged to 37°C one. For MTS test cells were separated again and incubated with 500 μ l Medium and MTS reagent (1:5) over 4 hours. Thereafter 2x 120 μ l were transferred into a 96 well plate and measured via SpectraMax 384.

2.4 Statistical analysis

For statistical analysis of co-culture and transwell experiments we calculated an average value from four different wells from each experiment and repeated each experiment 5 times. For each time point data were normalized to a control value at 37°C. Statistical analyses and graphs were created with GraphPad Prism version 5.0 software for Windows (GraphPad Software, San Diego CA, USA) and SPSS 18 (PASW Statistics 18, IBM, NY, USA). Comparison of the data was performed using one-way ANOVA. Comparison of Experiment 1 and 2 were performed using Lavene test, t-test and Mann-Whitney U-test. A value of $p < 0.05$ was considered as statistically significant.

2.5 Flow Cytometry

2.5.1 Intracellular staining protocol

LX-1 and HepG2 cells are stained in culture flasks 48 h before heating. 18 h before heating 5×10^5 cells are seeded in co-cultures (Collagen I 6-well Multiwell Plates, BD Biocoat, Germany) and induced with 2,5ng/ml TGF- β 1 (Figure 10).

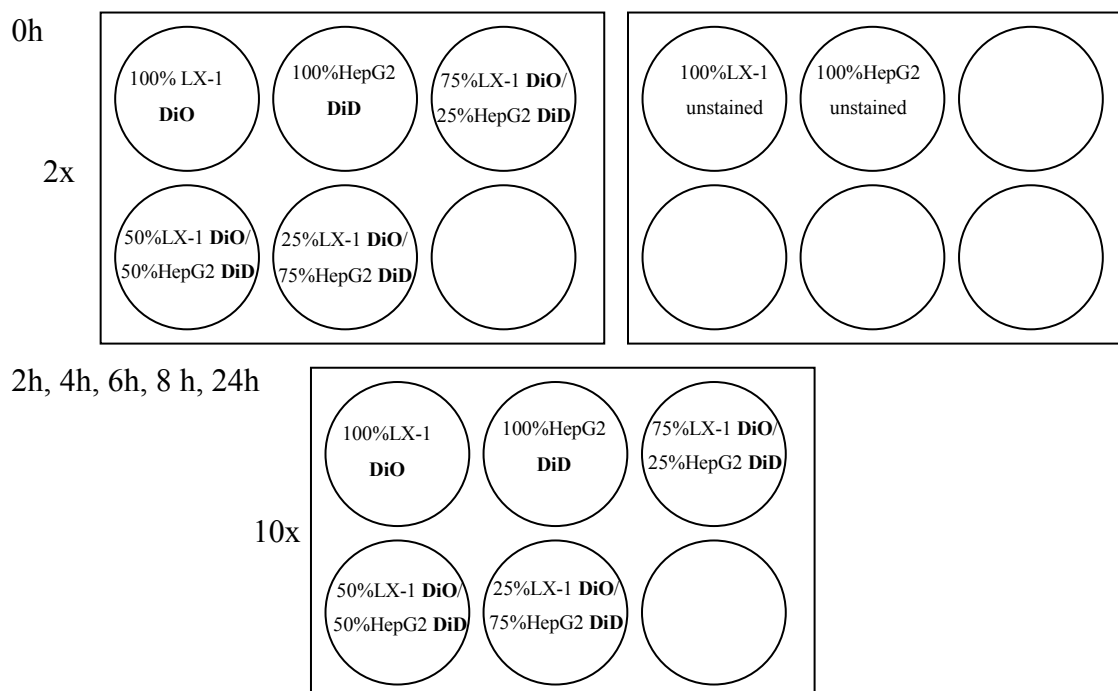


Figure 10: Scheme for seeding of HepG2 and LX-1 co-cultures 18 h before heating

Staining protocol for LX-1 cells:

The old medium was removed, then 4ml (in a 150cm² flask) staining medium (5µl DiO/1 ml medium) was added and incubated for 15 min at 37°C. Thereafter, the cells were washed 3 times by adding 12ml PBS and incubation of 10 min at 37°C.

Staining protocol for HepG2 cells:

The old medium was removed, then 2ml (in a 75cm² flask) staining medium (1µl DiD/1 ml medium) was added and incubated for 5 min at 37°C. Thereafter, the cells were washed 3 times by adding 6ml PBS and incubation of 10 min at 37°C.

2.5.2 Heating protocol

Immediately before heating the supernatant was withdrawn and 1ml preheated medium was added. The plates were incubated at desired temperature in preheated heating cabinet. Thereafter, the plates were cultivated at 37°C till use without changing the culture medium.

At each time point for heated cells and control the medium was removed and then the cells were washed with 1 ml PBS (collect medium and PBS in the same 15ml tube; conical tubes, BD, Germany). Thereafter the cells were detached with 200µl 0,025% Trypsin/ EDTA (Sigma-Aldrich, Germany) solution and incubate them at 37°C for 5-7 min. Then the cells were carefully resuspended with 1 ml medium and transfer cells into the 15ml tube.

2.5.3 Staining for Flow Cytometry measurement

For Flow Cytometry staining Annexin V PE and eFluor 450 was used. At first the 15ml falcon tube was centrifuged 350g for 5 min at room temperature. Then the supernatant was discard and the cells were washed with 1ml cold PBS. After another centrifugation (every time at 350g for 5 min) the supernatant was discard and the cells were resuspend in 1ml cold PBS. The cells were divided in two Flow Cytometry tubes (500µl each, Round-Bottom Tube, BD, Germany). After another centrifugation one pellet was solved in cold 500µl eFluor450 solution (dilution 1:15 in PBS; 7,5 ml PBS + 0,5 µl eFluor450 antibody), the other in 500µl cold PBS (unstained control) and incubated for 10 min on ice in the dark.

Subsequently, the cells were centrifuged and washed with 1ml cold PBS. After another centrifugation the supernatant was carefully removed and the cells were resuspend in 100µl 1x Annexin V dilution buffer (in PBS). In the first tube (already stained for

eFluor 450) 5µl Annexin V antibody were added and nothing into the second (control). After incubation at room temperature for 15min in the dark, 400µl 1x Annexin V dilution buffer were added. Then the samples were stored on ice and finally analysed by LSRII Flow Cytometer using FACSDiva 6.1.2 (BD Biosciences Europe, Germany).

2.5.4 Statistical analyses

The average of two or three experiments was calculated and graphs were created with GraphPad Prism version 5.0 software for Windows. Due to the lack of differences between the single cell lines in co-cultures an average of all data per cell line was used. Furthermore, the 37°C controls of each time point was set as one control data per experiment.

Table 2: Cell culture material and reagents

Reagent/Material	Company	Order number
Minimum Essential Medium (MEM)	Invitrogen, Austria	31095-029
Dulbecco's Modified Eagle Medium (DMEM)	PAA, Austria	E15-009
Foetal bovine serum (FBS)	PAA, Austria	A15-151
Penicillin/ Streptomycin (P/S)	PAA, Austria	P11-010
L-glutamine	PAA, Austria	M11-004
Trypsin-EDTA solution	Sigma-Aldrich, Germany	T4049
CellTiter 96® Aqueous One Solution Cell Proliferation Assay	Promega, Austria	G5430
Recombinant human TGF-β1	Biovision, USA	4342-5
Propidium iodide (PI)	BioChemika, Germany	No. 81845
Fluoresceindiacetate (FDA)	BioChemika, Germany	No. 31545
The Live/Dead® Viability/ Cytotoxicity Kit	Invitrogen, Austria	L-3224
Vybrant. DiO cell-labeling solution	Molekular Probes, Austria	V-22886
Vybrant. DiD cell-labeling solution	Molekular Probes, Austria	V-22887
PE Annexin V Apoptosis Detection Kit I	BD Biosciences Europe, Germany	559763
Fixable Viability Dye eFluor® 450	eBioscience, Austria	65-0863
Cell culture flasks 75cm ²	Corning, USA	3276/734-1544
Cell culture flasks 25cm ²	Corning; USA	3056/734-1532
Cell culture plates 96 well	Brand, Germany	781962
6-well Multiwell Plate	BD Biosciences Europe, Germany	353046
12 Well Cell Culture Plates	Corning; USA	3513
Polyester Membrane Transwell Clear Inserts	Corning; USA	3460
Collagen I 6-well Multiwell Plates	BD Biocoat, Germany	356400
Conical Tubes, 15ml	BD Biosciences Europe, Germany	352097
Round-Bottom Tubes	BD Falcon, Germany	352052

2.6 Animal experiments

All authorized animal experiments, including medication, anaesthesia and euthanasia, were carried out according to the Austrian law (ethical approval) and were performed by a veterinary anaesthesiologist.

2.6.1 Keeping of animals

The pigs were kept in boxes of two to four animals and fed twice a day with G-22 food for fattening (Gsellmann, Austria). The day of RFA as well as the day of euthanasia blood samples taken to control the condition and exclude illness or sepsis (Figure 11).



Figure 11: Pigs (*suus scrofa*) for RFA treatment standing in their box.

2.6.2 Anaesthesia

For the experiments pigs (n=19) with an approximate weight of 40 kg were used (Table 3). The animals were sedated with 10 mg diazepam, 120 mg azoperon and 500 mg ketamine. General anaesthesia was started with fentanyl 3 µg/kg bodyweight and 3 µg/kg bodyweight propofol as a bolus. Pigs were intubated and a bolus of 15 mg/kg bodyweight of cis-atracurium was administered. Volume controlled ventilation with a

minute volume of 8 ml/kg bodyweight was applied. Ventilation was adapted according to the results of blood gas analysis. Anaesthesia was continued with 0.08-0.1 µg/kg bodyweight/h remifentanyl and 2% sevoflurane. A central venous line was inserted for substitution of volume, blood pressure, oxygenation, ECG, temperature, capnometry, blood sugar and central venous pressure were monitored continuously.

Table 3: Pigs survived for different time spans after RFA

	Name	RFA date	Scarification date	Survival time	Fixation
III	C	15.04.09	15.06.09	2m	PF
III	D	15.04.09	15.06.09	2m	PF
III	E	13.05.09	16.06.09	1m	PF
III	F	13.05.09	15.06.09	1m	PF
III	G	24.06.09	18.09.09	3m	PF
III	H	24.06.09	18.09.09	3m	IF
III	I	26.01.10	09.02.10	2w	IF
III	K	26.01.10	09.02.10	2w	PF
III	M	29.03.10	30.03.10	1d	PF
III	N	19.04.10	18.05.10	1m	IF
III	O	05.05.10	06.07.10	2m	IF
III	P	17.05.10	19.05.10	2d	IF
III	Q	17.05.10	19.05.10	2d	PF
III	R	08.06.10	14.06.10	1w	PF
III	S	08.06.10	14.06.10	1w	IF
III	T	12.08.10	13.08.10	1d	IF
I	H	17.12.08	17.12.08	0d	IF
I	I	17.12.08	17.12.08	0d	IF
I	J	19.06.09	19.06.09	0d	PF

2.6.3 Radiofrequency ablation

The pig (n=19) was anaesthetised and transferred onto the CT (Aquilion One, Toshiba). Two electrodes (Rita Dispersive Electrode) were fixed on the pigs femoral (Table 4). For navigation the radiologist needed a topographic scan of the abdomen. For the selection of the right layer a 3mm abdominal scan was performed. After arrangement of the laser and the pig to the chosen layer, the radiologist scratched the skin with a scalpel. Then, he inserted the probe (Rita StarBurst XL, Electrosurgical Device)

through the skin into the liver during advised respiratory arrest. Three images were taken in a distance of 5mm around the chosen position (-5mm, 0mm und +5mm) also during respiratory arrest.

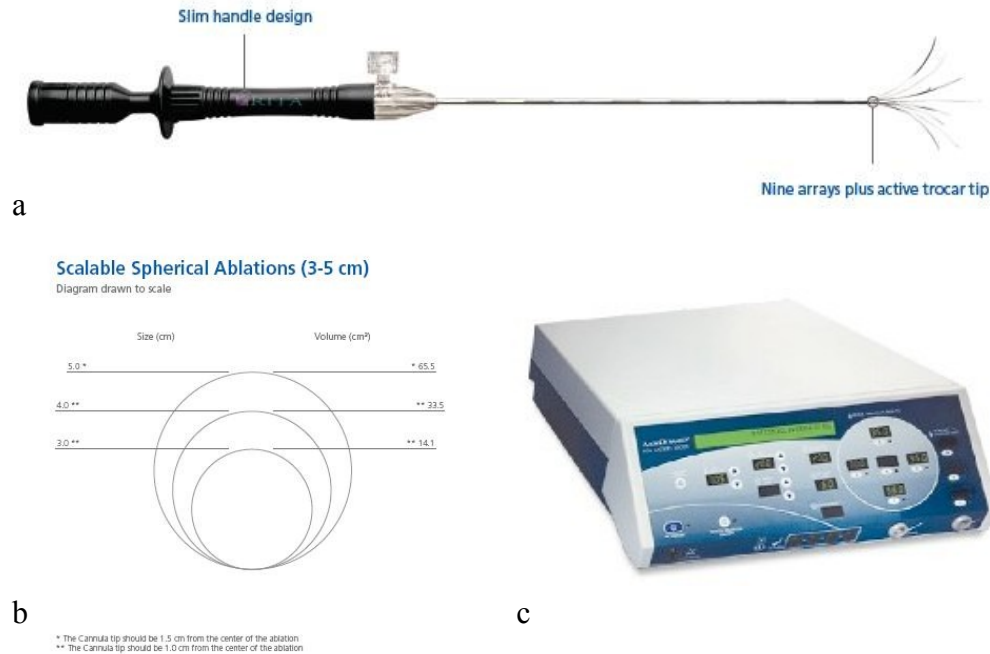


Figure 12: Rita probe and generator, scalable spherical ablations.

StarBurst XL RFA electrode (RITA, USA) (a) capable of creating spherical ablations in soft tissue from 3-5 cm in diameter (b) using the 1500X RF Generator (AngioDynamics, USA) (c)

The probe was placed to the final position, which could need further scans. Then the tips were extended to 3cm. The liver was scanned in 3 phases (arterial, portal venous and hepatic venous) using 40ml/kg contrast agent under respiratory arrest. The ablation (via ModelX RF Generator, Figure 12) lasted 7 minutes after reaching the target temperature of 100°C. Afterwards another 3 phase scan was performed, before the probe was placed for the second ablation. In the end a third 3 phase scan was done.

For the investigation of the wound healing response after RFA we treated in each case two pigs, which were euthanized at a certain date after treatment (immediately, one, two days, one, two weeks, one, two, three months). One pig from each date was used for large scale section and the other one for standard paraffin sections. Moreover, two lesions per pig were performed. One should be near a big vessel (central lesion) and the other one far away (peripheral lesion; Figure 14).

Table 4: Protocol for RFA for a single lesion

Ablation protocol	
1.	Fixation of the electrodes on pigs' femoral
2.	Selection of the ablation area (topography)
3.	Inserting the probe (under CT guidance)
4.	Expanding the tips to 3 cm
5.	3 phases scan
6.	RFA protocol (target temperature 100°C for 7 min)
7.	Removing the probe
8.	Final 3 phases scan



Figure 13: Preparation, ablation and liver explantation of a pig.

Sedation and blood collection of a pig in the pig sty by a veterinarian (a). RFA performed in the CT by the radiologist (b,c). Ablated liver tissue of a pig after thoracotomy in the animal operating room (d).

For **large scale sections** the liver has to be fixated by perfusion. Therefore, a thoracotomy and a median laparotomy were performed. Then the liver was mobilized without cutting the parenchyma or vessels. The common iliac arteries were dissected and entangled using a Vicryl 2 ligation. A third ligation was placed around the abdominal aorta. Furthermore, the vena cava inferior was dissected infrarenally as well

as supradiaphragmatically. Prior to application of heparin (10.000 IE), the aorta thoracalis was dissected above the diaphragma where an aortic clamp was placed. The iliac arteries were ligated and a canula flushed with 0.9% NaCl was placed into the aorta abdominalis and fixed using the ligature of the aorta as well as the right iliac artery. Then, the vena cava was cut infrarenally as well as supradiaphragmatically and a sucker was placed in the abdominal part. The aorta thoracalis was clamped and the abdominal organs were perfused with 3000 ml cold 0.9% NaCl in order to flush out the remaining blood. In the meantime, organ cooling was performed by rinsing cold 0.9% NaCl and crushed ice into the abdominal cavity. When all the blood was flushed out of the abdominal organs, perfusion was continued using formalin (4% Formalin/1X PBS) in order to fix the organs *in situ*.

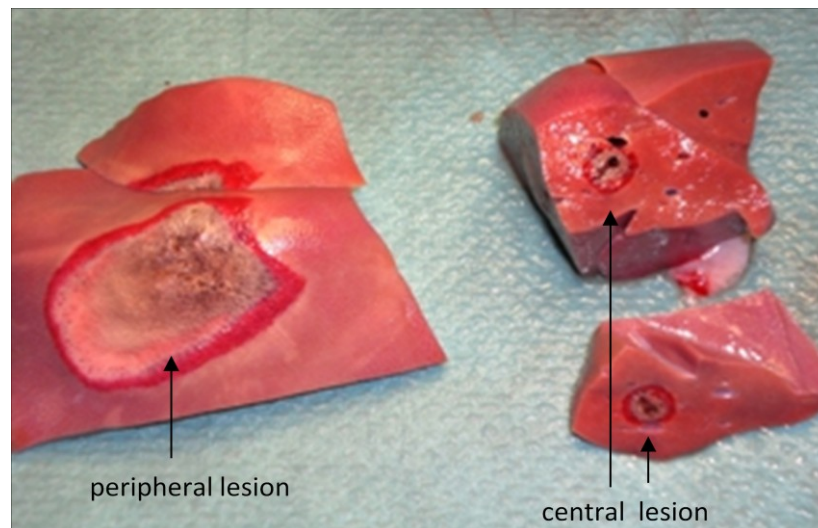


Figure 14: RFA area after perfusion fixation.

This picture displays differences between peripheral and central lesion. The central lesion is much smaller because of a cooling vessel nearby.

This method guarantees to keep the shape of the organs within the body while being dissected for further analysis. For preparation of the liver, the ligamentum hepatoduodenale was prepared and the biliary duct, the portal vein as well as the hepatic artery were ligated and cut. Then, the liver was dissected from the diaphragm without cutting the vena cava. After freeing the right as well as the left lobe of the organ, the liver is removed for back-table preparation. Finally, roughly the interesting parts of the liver were cut out using a knife and put into formalin until further processing (Figure 13d and Figure 14) [118].

For **standard paraffin blocks** after RFA a thoracotomy and a median laparotomy were performed to take the whole liver. Then the interesting parts were cut out using a knife and sliced into small pieces for further investigation.

2.6.4 Large scale sections

After removal of liver, the area of RFA was cut out. This area was cut into tissue blocks up to 5x5x3 cm in diameter. A single RFA area contained 1 to 3 tissue blocks. Dehydration of the blocks was done according to our optimized protocol: 3 days 4% Formalin, 12h 70% ethanol, 12h 90% ethanol, 12h 96% ethanol, 24-48 h 100% ethanol, 2-3 h acetone and 12h xylene. After dehydration the blocks were incubated in paraffin (melting point 52°C) for 18-24 h and then embedded in paraffin (melting point 56°C). The form for embedding was a custom product (by courtesy of Department of Obstetrics and Gynaecology), composed of 4 steel angle to vary the dimensions of the paraffin block. At intervals of 100µm, 2µm paraffin sections were performed. Sections were brought on 76 x 90 x 0,13mm object coverslips (Figure 15).

2.6.4.1 Histological staining

There was no need for processing the whole large paraffin block. Three serial sections were taken in defined distances and stained for chromotrope anilinblue (CAB), methylene blue (MB) and one for backup. After staining, sections were only dried, not covered by a coverslip. Then each section of the lesion was digitized.

~ CAB

Collagen fibres, reticular fibres and basal membranes are stained in blue. Nucleus is stained in violet and the cytoplasm in different nuances of blue. This staining helps to distinguish between vessels or artefacts. Also the coagulation of the surrounding area could be demonstrated.

Protocol: 0,5g anilinblue (Anstaltsapotheke) was solved in 99 ml warm distilled water, then were 2g chromotrope 2R (Anstaltsapotheke) added to the cold solution and finally 1ml 2N HCl was added to reach a pH of 1,8. The filtered, cold solution was ready for use.

First the sections were prewarmed in the incubator for 30 min at 37°C, then deparaffinized up to distilled water. Then, sections were stained with Haemalaun 3 min.

Thereafter, blueing with hot water was performed for 5 min. Prior of washing, sections were acid cleaned using 1% molybdato-phosphor-acid for 2 min. Then CAB solution was applied using a pipette to cover the plane lying section for 8 min. After incubation, the sections were washed in water for 1 min and dehydrated using an ascending alcohol row (50%, 70%, 90%, 100% ethanol and then 100% xylene).

~ *MB*

Methylene blue stains only in blue. The area of lesion is stained in a more intensive blue than the surviving cells around. This staining focuses on the difference of colour intensity between live and dead cells.

Protocol: First the sections were deparaffinized up to distilled water. After staining 1 min in methylenblue solution (1%; Anstaltsapotheke) sections were rinsed in distilled water. Then sections were washed in 90%, 100% ethanol, 100% xylene and then dried



Figure 15: Comparison of a large scale and a standard paraffin block and slide.

2.6.5 Standard paraffin sections

Interesting liver pieces put into embedding cassettes (Einbettkassetten #430711, Bio-Net, Sanowa, Germany) and fixated in 4% formalin. Overnight they were dehydrated by Tissue-Tek® Vacuum Infiltration Tissue Processor (Tissue-Tek® VIP®, Sakura,

USA) and paraffin embedded using Tissue-Tek® Tissue Embedding Console (Tissue-Tek® TEC™, Sakura, USA). For slicing the paraffin blocks were cooled on ice. 1.5-3µm thick sections were cut and transferred into a 45°C water bath and then mounted on adhesive StarFrost slides (ca. 76x26mm, cut edges; Knittel, Germany) and then air-dried.

Thereafter, the slices are stained for Caspase 3 (apoptosis), Hsp70 (heat stress), Ki67 (proliferation), Calprotectin (marker for macrophages and monocytes), GFAP (marker for activated hepatic stellate cells) and α -SMA (marker for myofibroblasts) for further characterization of the transition zone

2.6.5.1 Immunohistochemical staining protocols

UltraVision LP (HRP Polymer, Thermo Scientific, TL-125-HL) uses the technology of polymeric labelling. Polymer detection methods have been shown to provide increased sensitivity and detection simplicity. It is composed of smaller polymer subunits that minimize conflicts in binding the target protein. Decreased binding conflicts result in more consistent staining and better signal amplification. This results in a higher sensitivity and antibody efficiency. UltraVision detection system detects a specific mouse IgG or rabbit IgG antibody bound to an antigen in tissue sections. The specific antibody is located by a universal secondary antibody formulation conjugated to an enzyme-labelled polymer that recognizes mouse and rabbit immunoglobulins. The polymer complex is then visualized with an appropriate substrate or chromogen.

Components of TL-125-HL

1 Ultra V Block	(TA-125-UB)
1 Primary Antibody Enhancer	(TL-125-PB)
1 HRP Polymer	(TL-125-PH)

~ Activated Caspase 3

Caspases are involved in the initiation and execution of apoptosis. They are expressed as latent zymogens and are activated by an autoproteolytic mechanism or by processing by other proteases (frequently other caspases). Human caspases can be subdivided into three functional groups: cytokine activation (caspase-1, -4, -5, and -13), apoptosis initiation (caspase-2, -8, -9, and -10), and apoptosis execution (caspase-3, -6, and -7,

R&D Systems). Using this antibody for activated Caspase 3 we were able to distinguish apoptotic cells from non-apoptotic cells in immunohistochemistry.

Protocol: For staining activated Caspase 3 (R&D Systems, AF835) the UltraVision LP System was used. The antibody was dissolved in 500µl 1x PBS, aliquoted and frozen at 20°C.

The air-dried slides were heated for 30 min at 70°C in a heating cabinet. Then they were rehydrated and deparaffinized using xylene and isopropanol. After 2 times washing we retrieve the antigen epitopes using microwave. The slides were boiled in 0.1M Citrate buffer, pH 6.0 at 150 watt for 40 min. Subsequently, the slides were cooled down for 20 min to room temperature. Then they were washed 4 times in 1x PBS. The endogenous peroxidase was blocked for 15 min in 3% H₂O₂-Methanol at room temperature. Thereafter, we performed ultra UV-Blocking for 10 min. Without washing the slides we incubate them with the primary antibody in a dilution of 1:25 in Dako Antibody Diluent for 1 hour at RT. Then, the slides were washed 4 times in 1x PBS followed by incubation with Primary Antibody Enhancer for 10 min. After washing the slides 4 times in 1x PBS, they were incubated with HRP-Polymer for 30 min in the dark. Thereafter, the slides were washed 4 times in 1x PBS. For visualization the slides were incubated with AEC for 8 min. The reaction was stopped by washing with 1x PBS. Finally, the counterstaining was achieved with Hematoxylin and the slides coverslipped with Aquatex.

~ *Hsp70*

Heat shock proteins (HSPs) are involved in denaturation, folding, activation and transport of other proteins. They are synthesized under stressful conditions.

Protocol: For staining HSP70 (abcam, ab31010) the UltraVision LP System was used. The air-dried slides were heated for 30 min at 70°C in a heating cabinet. Then they were rehydrated and deparaffinized using xylene and isopropanol. After 2 times washing we retrieve the antigen epitopes using microwave. The slides were boiled in 0.1M Citrate buffer, pH 6.0 at 150 watt for 40 min. Subsequently, the slides were cooled down for 20 min to room temperature. Then they were washed 4 times in 1x PBS. The endogenous peroxidase was blocked for 15 min in 3% H₂O₂-Methanol at room temperature. Thereafter, we performed ultra UV-Blocking for 10 min. Without washing the slides we incubate them with the primary antibody in a dilution of 1:100 in Dako Antibody Diluent for 1 hour at RT. Then, the slides were washed 4 times in 1x

PBS followed by incubation with the Primary Antibody Enhancer for 10 min. After washing the slides 4 times in 1x PBS, they were incubated with HRP-Polymer for 30 min in the dark. Thereafter, the slides were washed 4 times in 1x PBS. For visualization the slides were incubated with AEC for 8 min. The reaction was stopped by washing with 1x PBS. Finally, the counterstaining was achieved with Hematoxylin and the slides coverslipped with Aquatex.

~ **Ki67**

This protein is expressed during late G1, S, G2 and M phases of the cell cycle, while in cells in G0 phase the protein cannot be detected. Normally it is localized in the nuclear matrix, but during mitosis it is present in all chromosomes. Therefore Ki-67 is a marker for cell proliferation. As positive control porcine lymph node tissue was used.

Protocol: For staining Ki67 Clone MIB-1 (Dako, M7240) the UltraVision LP System was used. The air-dried slides were heated for 30 min at 70°C in a heating cabinet. Then they were rehydrated and deparaffinized using xylene and isopropanol. After 2 times washing we retrieve the antigen epitopes using microwave. The slides were boiled in 0.1M Citrate buffer, pH 6.0 at 150 watt for 40 min. Subsequently, the slides were cooled down for 20 min to room temperature. Then they were washed 4 times in 1x PBS. The endogenous peroxidase was blocked for 15 min in 3% H₂O₂-Methanol at room temperature. Thereafter, we performed ultra UV-Blocking for 10 min. Without washing the slides we incubate them with the primary antibody in a dilution of 1:200 in Dako Antibody Diluent for 1 hour at RT. Then, the slides were washed 4 times in 1x PBS followed by incubation with Primary Antibody Enhancer for 10 min. After washing the slides 4 times in 1x PBS, they were incubated with HRP-Polymer for 30 min in the dark. Thereafter, the slides were washed 4 times in 1x PBS. For visualization the slides were incubated with DAB for 8 min. The reaction was stopped by washing with 1x PBS. Thereafter slides were counterstained with Hematoxylin and then dehydrated put in rising concentrations of isopropanol and xylene. Finally, the slides were coverslipped with GLC Mounting Medium.

~ ***GFAP (Thermo Scientific, MS-280-P0)***

This protein is a class-III intermediate filament. GFAP is a cell-specific marker that is expressed during the development of the central nervous system and can be used to distinguish astrocytes from other glial cells. In the literature it is described as marker for activated HSC [119, 120]. Porcine brain was used as positive control.

Protocol: For staining GFAP (Thermo Scientific, MS-280-P0) the UltraVision LP System was used. The air-dried slides were heated for 30 min at 70°C in a heating cabinet. Then they were rehydrated and deparaffinized using xylene and isopropanol. After 2 times washing we retrieve the antigen epitopes using microwave. The slides were boiled in 0.1M Citrate buffer, pH 6.0 at 150 watt for 40 min. Subsequently, the slides were cooled down for 20 min to room temperature. Then they were washed 4 times in 1x PBS. The endogenous peroxidase was blocked for 15 min in 3% H₂O₂-Methanol at room temperature. Thereafter, we performed ultra UV-Blocking for 10 min. Without washing the slides we incubate them with the primary antibody in a dilution of 1:500 in Dako Antibody Diluent for 1 hour at RT. Then, the slides were washed 4 times in 1x PBS followed by incubation with Primary Antibody Enhancer for 10 min. After washing the slides 4 times in 1x PBS, they were incubated with HRP-Polymer for 30 min in the dark. Thereafter, the slides were washed 4 times in 1x PBS. For visualization the slides were incubated with DAB for 8 min. The reaction was stopped by washing with 1x PBS. Thereafter slides were counterstained with Hematoxylin and then dehydrated put in rising concentrations of isopropanol and xylene. Finally, the slides were coverslipped with GLC Mounting Medium.

~ ***Calprotectin***

Calprotectin (S100A8/A9) has antibacterial, antifungal, immunomodulating and antiproliferative effects and is a potent chemotactic factor for neutrophils. Calprotectin can be detected in neutrophils, monocytes, macrophages and squamous mucosal epithelia and has been shown as an important marker for identifying macrophages in tissue sections (abcam, UK). As positive control porcine lymph node tissue was used.

Protocol: For staining Calprotectin-Monocyte/ Macrophage/ L1 Protein Ab-1 (Clone MAC 387) the UltraVision LP System was used. The air-dried slides were heated for 30 min at 70°C in a heating cabinet. Then they were rehydrated and deparaffinized using xylene and isopropanol. After 2 times washing we retrieve the antigen epitopes using 1mg/ml Trypsin (Calbiochem, Germany): The slides were incubated in a 37°C

waterbath for 15min. Then they were washed 4 times in 1x PBS. The endogenous peroxidase was blocked for 15 min in 3% H₂O₂-Methanol at room temperature. Thereafter, we performed ultra UV-Blocking for 10 min. Without washing the slides we incubate them with the primary antibody in a dilution of 1:500 in Dako Antibody Diluent for 1 hour at RT. Then, the slides were washed 4 times in 1x PBS followed by incubation with Primary Antibody Enhancer for 10 min. After washing the slides 4 times in 1x PBS, they were incubated with HRP-Polymer for 30 min in the dark. Thereafter, the slides were washed 4 times in 1x PBS. For visualization the slides were incubated with DAB for 8 min. The reaction was stopped by washing with 1x PBS. Thereafter slides were counterstained with Hematoxylin and then dehydrated put in rising concentrations of isopropanol and xylene. Finally, the slides were coverslipped with GLC Mounting Medium.

~ ***α-SMA***

This antibody stains the single isoform of α -smooth muscle actin and is chosen for detection of myofibroblasts. As positive control fibrotic porcine liver was used.

Protocol: For staining α -SMA antibody 1A4 (A2547, Sigma Aldrich) the UltraVision LP System was used. The air-dried slides were heated for 30 min at 70°C in a heating cabinet. Then they were rehydrated and deparaffinized using xylene and isopropanol. After 2 times washing we retrieve the antigen epitopes using microwave. The slides were boiled in 0.1M Citrate buffer, pH 6.0 at 150 watt for 40 min. Subsequently, the slides were cooled down for 20 min to room temperature. Then they were washed 4 times in 1x PBS. The endogenous peroxidase was blocked for 15 min in 3% H₂O₂-Methanol at room temperature. Thereafter, we performed ultra UV-Blocking for 10 min. Without washing the slides we incubate them with the primary antibody in a dilution of 1:5000 in Dako Antibody Diluent for 1 hour at RT. Then, the slides were washed 4 times in 1x PBS followed by incubation with Primary Antibody Enhancer for 10 min. After washing the slides 4 times in 1x PBS, they were incubated with HRP-Polymer for 30 min in the dark. Thereafter, the slides were washed 4 times in 1x PBS. For visualization the slides were incubated with AEC for 8 min. The reaction was stopped by washing with 1x PBS. Finally, the counterstaining was achieved with Hematoxylin and the slides were coverslipped with Aquatex.

2.6.5.2 Antibodies and other reagents

Xylene, isopropanol, Citrate buffer, 1X PBS, Hematoxylin was provided from Anstaltsapotheke, Medical University Graz.

Table 5: Antibodies and other reagents used for immnohistochemistry

Name	Specifity	Company	Host	Reference number	Dilution	Localisation
Active Caspase 3	human/ mouse	R&D Systems, Germany	rabbit	AF835, Lot CFZ31, 50µg	1:25	Cytoplasm
Hsp70 (heat shock protein 70)	human	Abcam, UK	rabbit, pc	ab31010	1:100	Cytoplasm
Ki-67	human	Dako, Austria	mouse, mc	M7240, Clone MIB-1	1:200	Nucleus
GFAP (Glial fibrillary acidic protein)	human, pig,..	Thermo Scientific, Belgium	mouse, mc	MS-280-P0, Clone GA-5	1:500	Cytoplasm
Calprotectin (Monocyte/ Macrophage/ L1 Protein Ab-1)	human, pig,..	Thermo Scientific, Belgium	mouse, mc	MS-148-P0; Clone MAC 387	1:500	Cytoplasm
α-SMA (alpha-smooth muscle actin)	human, mouse	Sigma Aldrich, Germany	mouse mc	A 2547, Clone 1A4	1:5000	Cytoplasm
Dako REAL™ Antibody Diluent		Dako, Austria		S2022, 250 ml, Lot 00028133		
UltraVision LP	mouse/ rabbit	Thermo Scientific, Belgium		TL-125-HL		
Trypsin		Calbiochem, Germany	bovine, pancreas	6502	1 mg/ml	
AEC		Dako, Austria		K346430		
DAB		Dako; Austria		K346811		
Aquatex®		Merck, Germany		108562		
GLC Mounting Medium		Sakura Finetek, Netherlands		1408		

mc.. monoclonal, pc.. polyconal,

3 Results

3.1 Co-culture experiments with MRC-5 and HepG2 cells

HepG2 and MRC-5 cells were seeded together followed by heating from 55°C to 100°C for 5 to 15 min. Moreover, survival of MRC-5 cells, HepG2 cells and their co-cultures were documented immediately, as well as 24 h and 48 h after heat treatment (Table 6 and Figure 16).

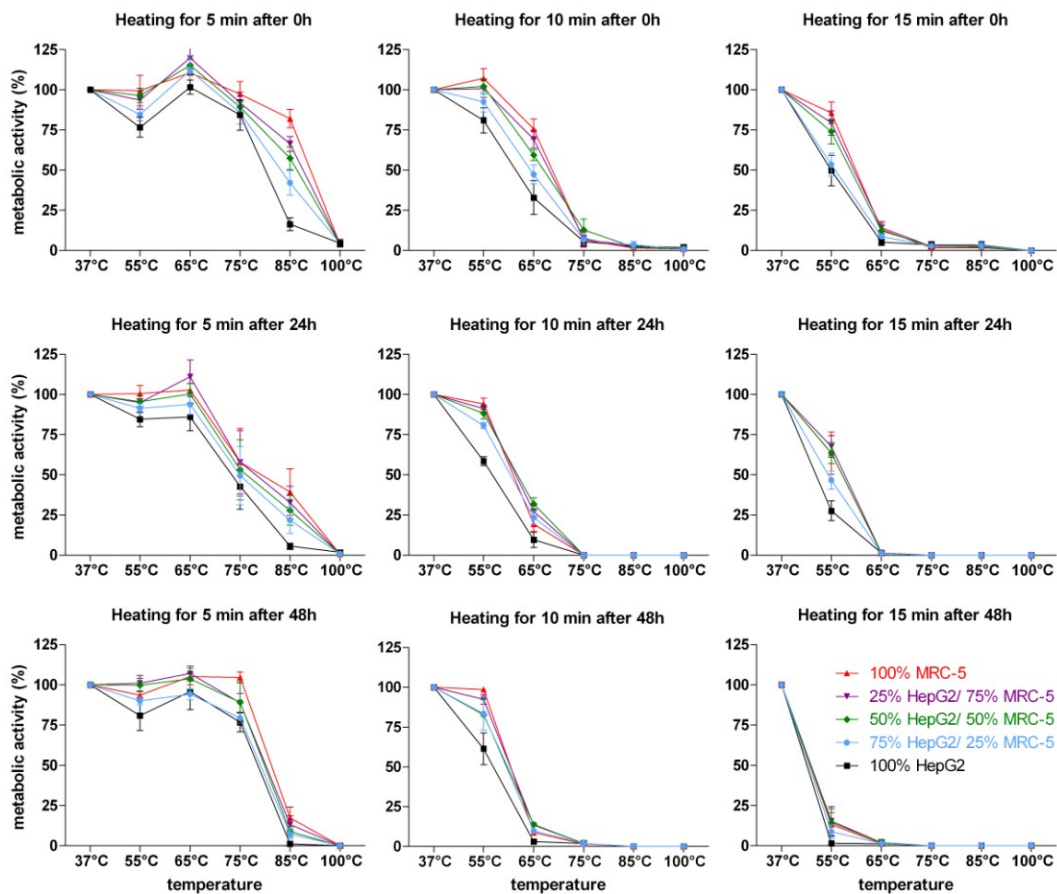


Figure 16: Shift of viabilities after heating experiments ranging from 5 - 15 minutes.

Heating the cell lines HepG2, MRC-5 cells and their co-cultures of 25% HepG2 and 75% MRC-5 (25%Hep/75%MRC), 50% HepG2 and MRC-5 (50%Hep/50%MRC) and 75% HepG2 and 25% MRC-5 (75%Hep/25%MRC) at 55°C, 65°C, 75°C, 85°C and 100°C for 5 to 15 minutes. Metabolic activity was tested either immediately (a-c) after exposure to the different temperatures, after 24 h (d-f) as well as after 48 h (g-i) of cell culture. The data were determined using the MTS-test and then normalized to the exposure at 37°C (100%). Comparison of the data was performed using one-way ANOVA.

Table 6: Percentages of viable HepG2 and MRC-5 cells in heating experiments.

Data were generated by heating the cell lines HepG2, MRC-5 and their co-cultures at 55°C, 65°C, 75°C, 85°C and 100°C. The co-cultures consist of different amounts of cells of the two cell lines: 25%Hep/75%MRC, 50%Hep/50%MRC, 75%Hep/25%MRC. Viability was tested either immediately after exposure to the different temperatures for different time spans (5, 10 or 15 minutes), after 24 h as well as after 48 h of cell culture. The data were determined using the MTS-test and normalized to the exposure at 37°C (100%). The table shows the percentage of metabolic activity (first column) and the standard error (second column) after heating. 5' 0 h: viability measurement immediately after exposure to the different temperatures for 5 minutes; 5' 24 h viability measurement after 24 h, etc. The average of cell viability and the standard error were calculated from 6 repeated tests.

°C	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
5' 0 h	HepG2		MRC-5		25% HepG2		50% HepG2		75% HepG2	
55°C	76,66	6,23	99,52	9,37	93,62	5,76	96,52	4,42	84,6	3,99
65°C	101,65	4,41	110,57	8,63	119,75	6,5	115,24	6,07	111,92	9,62
75°C	84,34	9,57	97,35	7,76	91,83	6,65	89,24	3,81	85,56	6,79
85°C	16,4	3,91	82,08	5,69	66,39	4,63	57,41	7,01	42,15	7,59
100°C	4,68	2,22	3,85	1,64	4,15	1,73	4,06	1,66	4,41	1,82
5' 24 h	HepG2		MRC-5		25% HepG2		50% HepG2		75% HepG2	
55°C	84,68	4,57	100,58	4,93	94,9	2,46	95,46	3,73	91,4	2,93
65°C	86,06	8,69	102,72	4,18	111	10,48	100,22	6,51	93,84	6,16
75°C	42,77	14,12	57,83	19,54	57,87	20,95	53,13	18,74	49,55	18,13
85°C	5,64	1,91	39,32	14,49	32,78	10,1	27,84	9,25	21,84	8,28
100°C	1,78	1,78	0,66	0,66	0,8	0,8	0,88	0,88	0,86	0,86
5' 48 h	HepG2		MRC-5		25% HepG2		50% HepG2		75% HepG2	
55°C	80,92	9,33	93,6	2,14	101	4,73	99,88	4,13	90,1	2,38
65°C	95,89	11,35	105,2	5,17	107,05	4,58	103,83	6,57	94,2	3,48
75°C	76,7	5,8	104,67	3,33	88,8	5,91	89,13	12,99	79,47	0,99
85°C	1,21	1,01	17,3	6,82	13,2	5,37	8,77	3,14	7,3	2,93
100°C	0	0	0	0	0	0	0	0	0	0
10' 0 h	HepG2		MRC-5		25% HepG2		50% HepG2		75% HepG2	
55°C	80,96	7,88	107,18	6,09	100,77	5,56	102,09	4,65	92,57	6,25
65°C	32,96	10,48	75,61	6,23	69,33	5,49	59,41	3,38	47,39	5,91
75°C	5,61	2,3	6,7	4,61	7,52	4,81	12,85	6,7	6,78	3,01
85°C	2,85	1,75	1,66	1,02	2,03	1,25	2,2	1,35	3,53	2,28
100°C	1,92	1,92	1,04	1,04	0,94	0,94	1,06	1,06	1	1
10' 24 h	HepG2		MRC-5		25% HepG2		50% HepG2		75% HepG2	
55°C	58,58	2,7	94,12	3,55	91,15	2,15	88,27	3,51	80,73	1,88
65°C	9,63	4,84	19,44	4,74	27,28	4,04	31,9	3,91	23,38	4,16
75°C	0	0	0	0	0	0	0	0	0	0
85°C	0	0	0	0	0	0	0	0	0	0
100°C	0	0	0	0	0	0	0	0	0	0
10' 48 h	HepG2		MRC-5		25% HepG2		50% HepG2		75% HepG2	
55°C	61,43	10,02	98,58	1,16	92,53	3,08	82,73	9,57	83,47	10,33
65°C	3,06	1,08	8,85	1,44	13,38	1,04	13,78	0,55	9,7	1,65
75°C	1,89	1,89	1,63	1,63	1,87	1,87	1,97	1,97	1,97	1,97
85°C	0	0	0	0	0	0	0	0	0	0

100°C	0	0	0	0	0	0	0	0	0	0
15' 0 h	HepG2		MRC-5		25% HepG2		50% HepG2		75% HepG2	
55°C	49,77	9,51	85,71	6,74	79,39	7,56	74,02	7,71	53,52	7,16
65°C	5,21	1,96	13,21	3,86	14,23	3,79	12,35	3,25	8,76	1,71
75°C	3,44	2,27	1,77	1,06	2,55	1,53	2,67	1,6	2,83	1,74
85°C	3,39	2,34	1,68	1,1	2,4	1,62	2,14	1,36	2,96	1,95
100°C	0	0	0	0	0	0	0	0	0	0
15' 24 h	HepG2		MRC-5		25% HepG2		50% HepG2		75% HepG2	
55°C	27,63	6,13	63,47	13,07	67,63	6,7	63,6	6,56	46,67	5,72
65°C	1,4	0,71	1,28	0,6	1,48	0,55	1,3	0,52	1,18	0,46
75°C	0	0	0	0	0	0	0	0	0	0
85°C	0	0	0	0	0	0	0	0	0	0
100°C	0	0	0	0	0	0	0	0	0	0
15' 48 h	HepG2		MRC-5		25% HepG2		50% HepG2		75% HepG2	
55°C	1,53	1,53	13,13	7,42	15,33	8,95	14,4	8,65	8,67	4,34
65°C	1,3	1,3	1,45	1,45	1,98	1,98	1,9	1,9	1,53	1,53
75°C	0	0	0	0	0	0	0	0	0	0
85°C	0	0	0	0	0	0	0	0	0	0
100°C	0	0	0	0	0	0	0	0	0	0

The results showed highly significant differences between the heat resistance of liver carcinoma cells and fibroblasts. HepG2 cells were most sensitive to heat-induced cell death and the sensitivity decreased with rising percentages of MRC-5 cells in the co-culture. Figure 16 compiles the shift of metabolic activity of HepG2 and MRC-5 cells focusing on different temperatures.

Heating MRC-5 und HepG2 cells at 100°C for 5 min led immediately to cell death (Figure 16) in contrast to heating at 85°C, which induces cell death over 48 h. The cells were not permanently damaged after heating at 55°C, 65°C and 75°C for 5 min.

Heating MRC-5 und HepG2 cells at 100°C, 85°C and 75°C for 10 min caused immediate cell death in contrast to 65°C, where cells died within 48 h. Heat exposure at 55°C damaged the cells not permanently except 100% HepG2, which showed a clearly lower metabolic activity compared to other co-cultures.

Heating MRC-5 und HepG2 at 100°C, 85°C, 75°C and 65°C for 15 min led immediately to cell death. Cells heated at 55°C showed a distinct decreasing of the metabolic activity over 48 h until only 0-25% of all cells were viable.

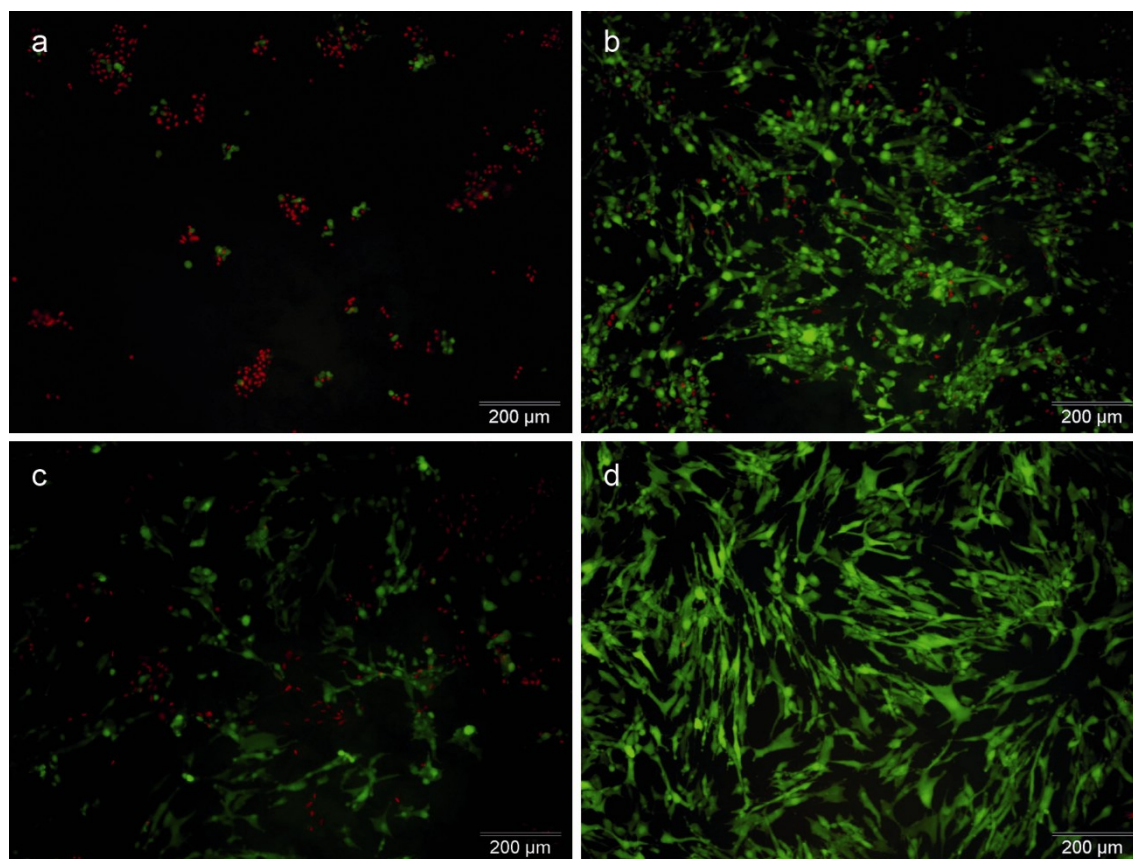


Figure 17: Fluorescence staining of cell viability.

HepG2, MRC-5 cells and their co-cultures of 25% HepG2 and 75% MRC-5 (25%Hep/75%MRC), 50% HepG2 and MRC-5 (50%Hep/50%MRC) and 75% HepG2 and 25% MRC-5 (75%Hep/25%MRC) were heated for 10 minutes at 65°C in a 24 well plate and then stained for Fluorescein diacetate (FDA; viable cells: green) and Propidium Iodide (PI; dead cells: red). The pictures were taken using an inverted Olympus IX51 microscope. A green filter was used for visualization of PI (red) with wavelengths from 520 to 550nm. For FDA (green) a blue filter was used with wavelengths from 460 to 490nm. After heating almost all HepG2 cells (a) are dead in contrast to MRC-5 cells (d), which are mostly viable. Pictures show mostly dead HepG2 cells in co-culture of 50%Hep/50%MRC (c) and 25%Hep/75%MRC (b) (x 10 magnification).

3.1.1 Cell survival after temperature exposure for 5min

After heating MRC-5, HepG2 cells and their co-cultures at 55°C, 65°C and 75°C we found no statistically significant differences, but a trend that HepG2 cells were more heat sensitive (Figure 18 and Table 6) than MRC-5 cells.

100% MRC-5 cells compared to co-cultures with 25%Hep/75%MRC ($p < 0.001$) cells and 50%Hep/50%MRC ($p < 0.01$) cells were significantly different in terms of cell metabolic activity after heat exposure for 5 minutes at 85°C.

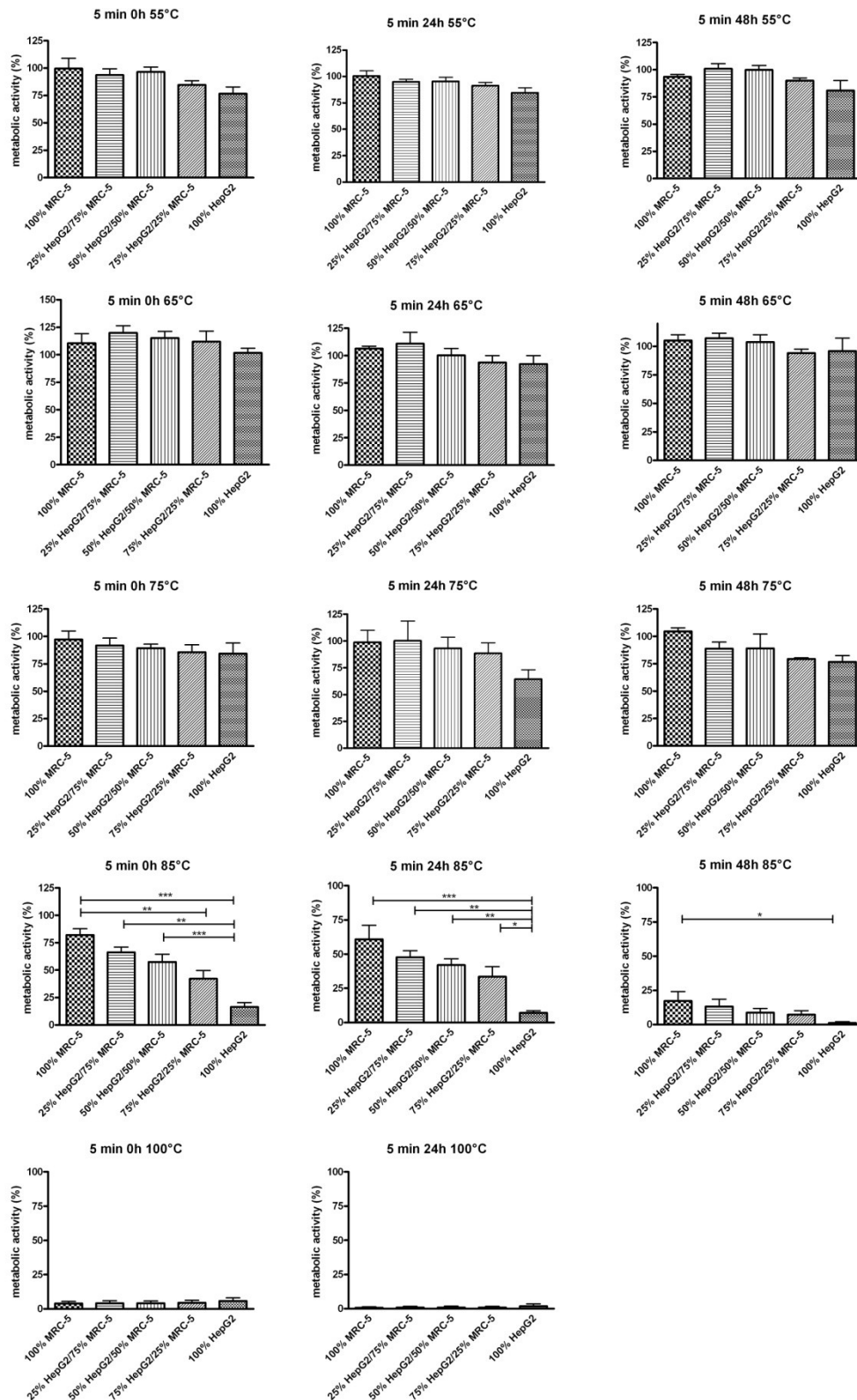


Figure 18: Heating of MRC-5, HepG2 cells and their co-cultures for 5 minutes.

Heating HepG2 and LX-1 cells for 5 min at 55°C, 65°C and 75°C displayed marginal differences between metabolic activity of HepG2 and MRC-5 cells. The metabolic activity of HepG2 cells was immediately after heating at 85°C and over 48 h significantly lower compared to MRC-5 cells. Heating the cells at 100°C killed immediately nearly all cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Furthermore, co-cultures of 25%Hep/75%MRC cells showed significantly higher metabolic activity compared to 75%Hep/25%MRC cells ($p<0.01$) and 100% HepG2 ($p<0.001$). After 24 h the metabolic activity of 100% HepG2 cells was significantly lower compared to 100% MRC-5 ($p<0.001$), 25%Hep/75%MRC ($p<0.01$), 50%Hep/50%MRC ($p<0.01$) and 75%Hep/25%MRC cells ($p<0.05$). 48 hours after heating for 5 minutes at 85°C the cells, we still found a significant difference between 100% MRC-5 and 100% HepG2 ($p<0.05$).

After heating at 100°C for 5 min almost all cells died and no significant differences were detected regarding the different co-cultures.

3.1.2 Cell survival after temperature exposure for 10 min

Heating up for 10 min at 55°C (Figure 19 and Table 6) showed immediately no significant differences in metabolic activity. After 24 h the metabolic activity of 100% MRC-5 ($p<0.001$), 25%Hep/75%MRC ($p<0.001$), 50%Hep/50%MRC ($p<0.001$) and 75%Hep/25%MRC cells ($p<0.01$) compared to 100% HepG2. Furthermore, we found that the metabolic activity of 25%Hep/75%MRC differs significantly from 75%Hep/25%MRC cells ($p<0.05$).

After 48 h at heating for 10 min at 55°C the metabolic activity of 100% HepG2 cells was significantly lower compared to 100% MRC-5 ($p<0.001$), 25%Hep/75%MRC ($p<0.01$), 50%Hep/50%MRC ($p<0.01$) and 75%Hep/25%MRC cells ($p<0.05$). After heating up for 10 min at 65°C the metabolic activity of 100% HepG2 cells was significantly lower compared to 100% MRC-5 ($p<0.01$) and 25%Hep/75%MRC cells ($p<0.01$).

After heating for 10 minutes at 65°C the metabolic activity of 100% HepG2 cells was significantly lower compared to 100% MRC-5 ($p<0.01$) and 25%Hep/75%MRC ($p<0.01$). Additionally, we found significant differences comparing 100% MRC and 75%Hep/25%MRC cells ($p<0.05$). After 24 h the metabolic activity of 50%Hep/50%MRC cells was significantly higher compared to 100% HepG2 cells ($p<0.05$).

After heating at 75°C for 10 minutes almost all cells died and no significant differences were detected regarding the different co-cultures. Heating up at 85°C and 100°C displayed survival less than 4% of each cell line for all experimental groups

immediately after heat exposure and cells did not recover overnight and after 48 h of cell culture at 37°C.

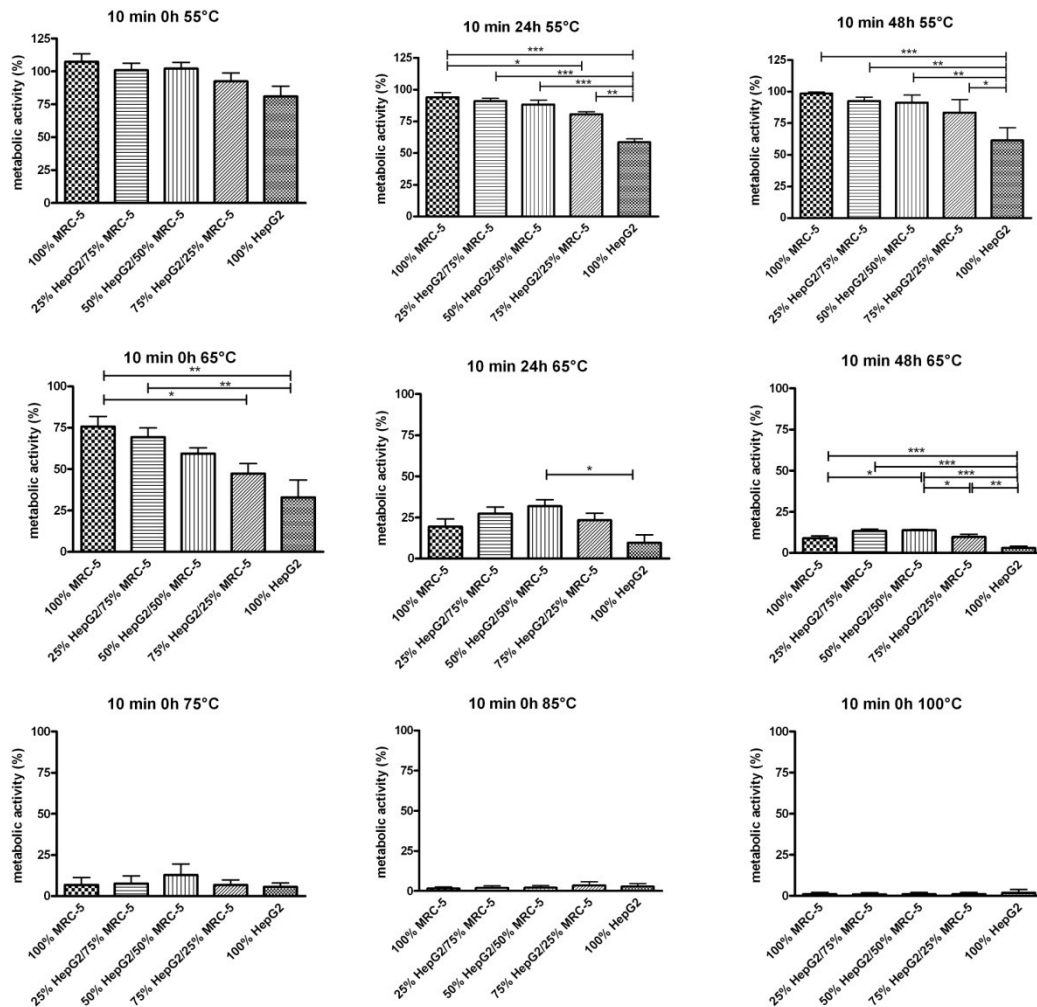


Figure 19: Heating of MRC-5, HepG2 cells and their co-cultures for 10 minutes.

Heating HepG2 and MRC-5 cells for 10 min at 55°C displays a partly significantly greater heat sensitivity of HepG2 cells. The metabolic activity of HepG2 cells was immediately after heating at 65°C for 10 min significantly lower compared to MRC-5 cells. Within 48 h all cells except 15% died. Heat exposure at 75°C killed within 24 h all cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.1.3 Cell survival after temperature exposure for 15 minutes

Heating for 15 min at 55°C (Figure 20 and Table 6) showed immediately no significant differences. 24 h after heating the metabolic activity of 100% HepG2 cells was significantly lower compared to 100% MRC-5 ($p < 0.05$), 25%Hep/75%MRC ($p < 0.05$) and 50%Hep/50%MRC cells ($p < 0.05$).

Temperature exposure at 65°C, 75°C, 85°C as well as 100°C for 15 min showed no statistically significant differences in terms of metabolic activity for all experimental groups.

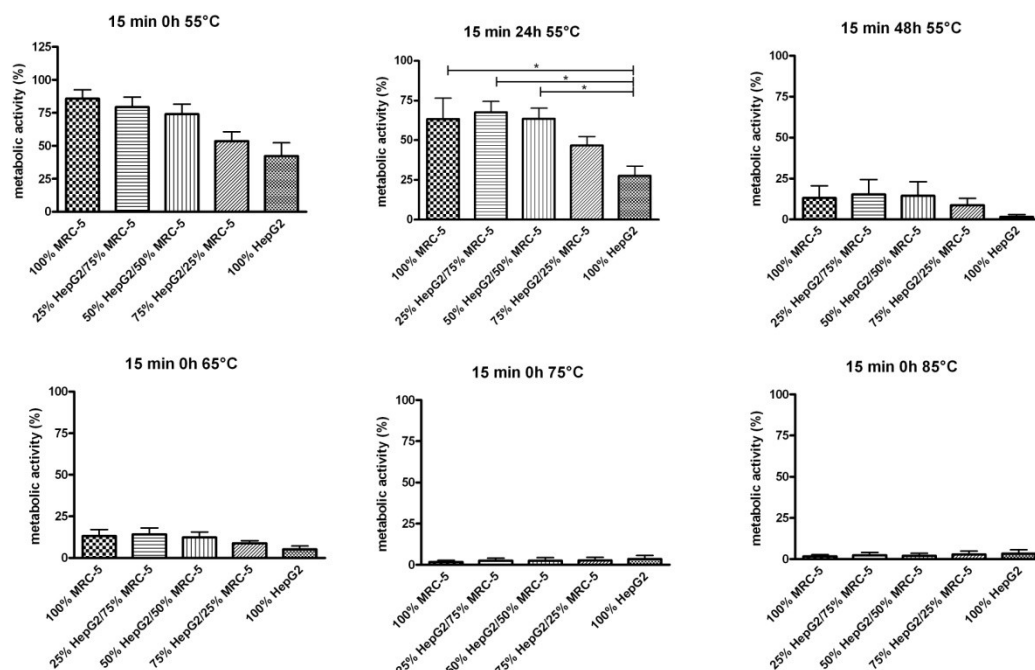


Figure 20: Heating of MRC-5, HepG2 cells and their co-cultures for 15 minutes.

Heating HepG2 and MRC-5 cells for 15 min at 55°C displays a partly significantly greater heat sensitivity of HepG2 cells. Heat exposure 65°C killed over 48 h nearly all cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.2 Co-culture experiments with LX-1 and HepG2 cells

The effect of heat exposure using different temperatures (55°C, 65°C, 75°C, 85°C) and different time spans (5 min, 10 min and 15 min) on the liver carcinoma cells HepG2 and stellate cells LX-1 and their co-cultures (25%HePG2/75%LX-1, 50%HePG2/50%LX-1 and 25%HePG2/75%LX-1) were determined. Moreover, survival of LX-1 cells, HepG2 cells and their co-cultures were documented immediately, 24 h and 48 h after heat treatment (Table 7). Figure 21 compiles the shift of metabolic activity of HepG2 and LX-1 cells focusing on different temperatures.

Heating LX-1 und HepG2 cells at 100°C for 5 min caused immediate cell death (Figure 21) in contrast to heating at 85°C, which induced cell death over 48 h. The cells were not permanently damaged after heating at 55°C, 65°C and 75°C for 5 min.

Heating LX-1 und HepG2 cells at 100°C, 85°C and 75°C for 10 min led immediately to cell death in contrast to heating at 65°C, which kills the cells within 48 h. Heat exposure at 55°C did not damage the cells permanently except 100% HepG2, which show a clearly lower metabolic activity compared to other co-cultures.

Heating LX-1 und HepG2 cells at 100°C, 85°C, 75°C and 65°C for 15 min led immediately to cell death. Cells heated at 55°C showed a distinct decreasing of the metabolic activity over 48 h to only 0-25% of all cells were viable.

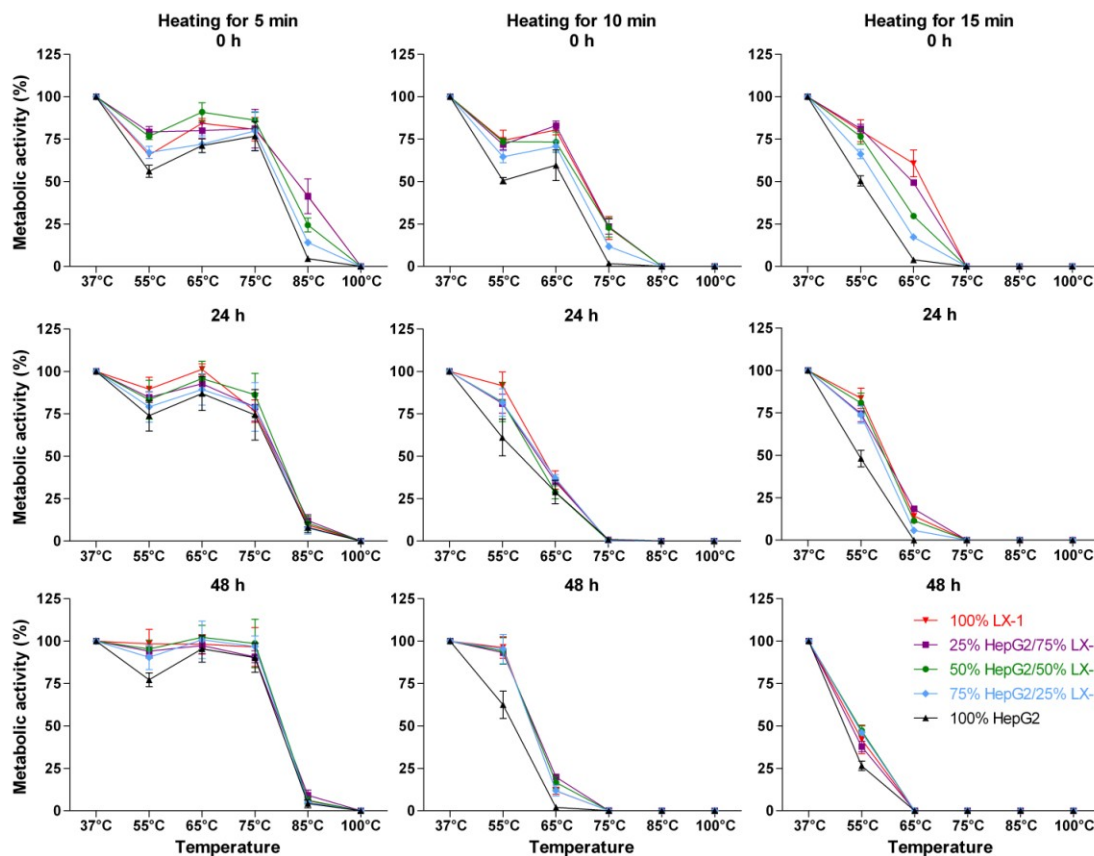


Figure 21: Shift of metabolic activity after hyperthermia ranging from 5 - 15 minutes.

Data were generated by heating the cell lines HepG2, LX-1 and their co-cultures at 55°C, 65°C, 75°C and 85°C. Metabolic activity was tested either immediately after exposure to the different temperatures for different time spans (5, 10 or 15 minutes), after 24 h as well as after 48 h of cell culture. The data were determined using the MTS-test and normalized to the exposure at 37°C (100%). Comparison of the data was performed using one-way ANOVA.

3.2.1 Cell survival after temperature exposure for 5 min

After 24 h the metabolic activity was significantly different between 100% LX-1 and 100% HepG2 cells ($p < 0.05$; Figure 23). After 48 h we could verify that the metabolic activity of 100% HepG2 was significant lower than 100% LX-1 ($p < 0.01$), 75% HepG2/

25% LX-1 ($p<0.05$), 50% HepG2/ 50% LX-1 ($p<0.01$) and 25% HepG2/ 75% LX-1 cells ($p<0.01$). Heat exposure for 5 min at 65 showed only immediately significant differences between 50% HepG2/ 50% LX-1 and 100% HepG2 cells, but not after 24 and 48 h. Just as heating cells for 5 min at 75°C and 85°C.

Table 7: Percentages of viable HepG2 and LX-1 cells in heating experiments.

Data were generated by heating the cell lines HepG2, LX-1 and their co-cultures at 55°C, 65°C, 75°C, 85°C and 100°C. Viability was tested either immediately after exposure to the different temperatures for different time spans (5, 10 or 15 minutes), after 24 h as well as after 48 h of cell culture. The data were determined using the MTS-test and normalized to the exposure at 37°C (100%). The table shows the percentage of metabolic activity (first column) and the standard error (second column) after heating. 5' 0 h: viability measurement immediately after exposure to the different temperatures for 5 minutes; 5' 24 h viability measurement after 24 h, etc. The average of cell viability and the standard error were calculated from 6 repeated tests.

°C	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
5' 0 h	HepG2		LX-1		HepG2 25%		HepG2 50%		HepG2 75%	
55°C	56,06	3,56	75,75	10,11	77,98	4,041	76,69	1,867	67,18	3,628
65°C	71,14	3,977	84,44	2,809	80,03	4,458	91,04	5,489	71,98	4,999
75°C	76,82	17,18	80,85	6,92	81,27	11,39	86,19	4,70	79,83	11,45
85°C	4,612	0,301	41,49	1,557	41,35	10,30	24,40	4,06	14,09	0,989
10' 0 h	HepG2		LX-1		HepG2 25%		HepG2 50%		HepG2 75%	
55°C	50,54	1,874	74,37	5,866	69,11	3,547	73,53	1,823	64,67	3,519
65°C	59,64	8,994	80,4	2,871	82,47	2,701	73,31	6,065	70,74	3,338
75°C	1,68	0,98	22,73	6,73	23,51	4,46	22,95	5,57	11,77	1,69
15' 0 h	HepG2		LX-1		HepG2 25%		HepG2 50%		HepG2 75%	
55°C	50,5	3,035	79,95	6,444	79,43	3,032	76,57	4,404	66,25	2,809
65°C	3,94	0,73	60,72	7,86	17,39	49,44	0,13	0,15	17,39	1,01
5' 24 h	HepG2		LX-1		HepG2 25%		HepG2 50%		HepG2 75%	
55°C	57,89	9,25	89,64	6,99	82,85	4,66	83,41	11,46	79,22	9,08
65°C	88,95	11,01	101,3	3,216	89,43	9,241	95,89	10,08	92,77	3,39
75°C	74,46	14,97	78,58	5,61	79,06	8,30	86,29	12,60	73,12	11,53
85°C	8,42	3,00	9,50	3,22	12,10	3,62	10,58	4,287	7,647	3,434
10' 24 h	HepG2		LX-1		HepG2 25%		HepG2 50%		HepG2 75%	
55°C	61,08	10,82	91,57	8,03	82,32	5,01	82,08	11,64	81,70	8,13
65°C	28,98	6,86	36,10	5,42	35,20	0,10	28,84	3,90	37,00	2,19
75°C	0,98	0,53	0,13	0,07	0,71	0,15	0,48	0,07	0,27	0,25
15' 24 h	HepG2		LX-1		HepG2 25%		HepG2 50%		HepG2 75%	
55°C	48,14	4,85	83,71	5,94	77,39	5,80	77,85	6,03	73,94	5,04
65°C	0,02	0,02	14,22	3,80	18,46	0,67	11,47	1,08	5,75	0,10
5' 48 h	HepG2		LX-1		HepG2 25%		HepG2 50%		HepG2 75%	
55°C	60,21	3,18	98,46	8,46	93,16	2,82	95,39	5,19	90,47	7,18
65°C	95,46	7,82	98,18	5,71	97,48	4,85	102,20	7,16	100,80	11,01
75°C	90,29	8,631	96,59	11,52	96,84	6,20	98,72	14,29	90,83	3,51
85°C	4,292	2,419	5,938	1,624	9,23	3,05	6,19	2,12	4,92	1,31
10' 48 h	HepG2		LX-1		HepG2 25%		HepG2 50%		HepG2 75%	
55°C	62,54	7,995	96,26	6,35	93,23	1,59	94,22	7,77	95,35	8,67
65°C	1,98	1,86	11,86	2,07	11,93	3,11	16,67	3,61	19,97	1,41
75°C	0,099	0,099	0,013	0,013	0,14	0,01	0	0	0,149	0,099
15' 48 h	HepG2		LX-1		HepG2 25%		HepG2 50%		HepG2 75%	
55°C	26,55	2,788	42,07	8,38	37,87	3,14	47,20	2,82	46,07	1,28
65°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00

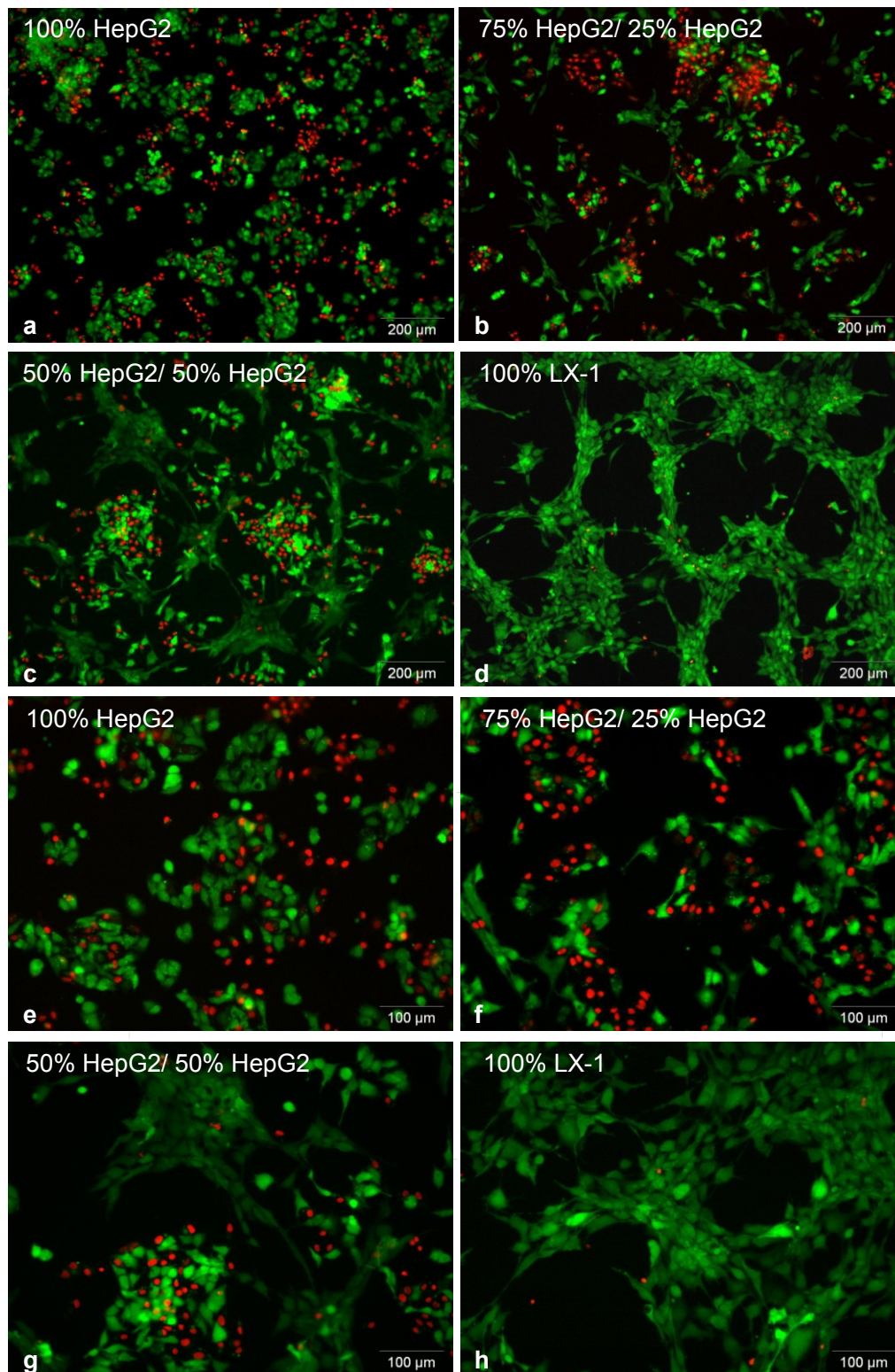


Figure 22: Differences in cell death induced via hyperthermia.

Pictures showed differences in live and death staining (Live/Dead® Viability/Cytotoxicity Kit) after heating LX-1, HepG2 and their co-cultures for 10 min at 65°C. HepG2 (a, e) are much more sensible to hyperthermia than LX-1 (d, h). This could also be shown in co-cultures metabolic activity decreased with the rising percentage of HepG2 cells in co-cultures (b, c, f, g). 10x and 20x magnification

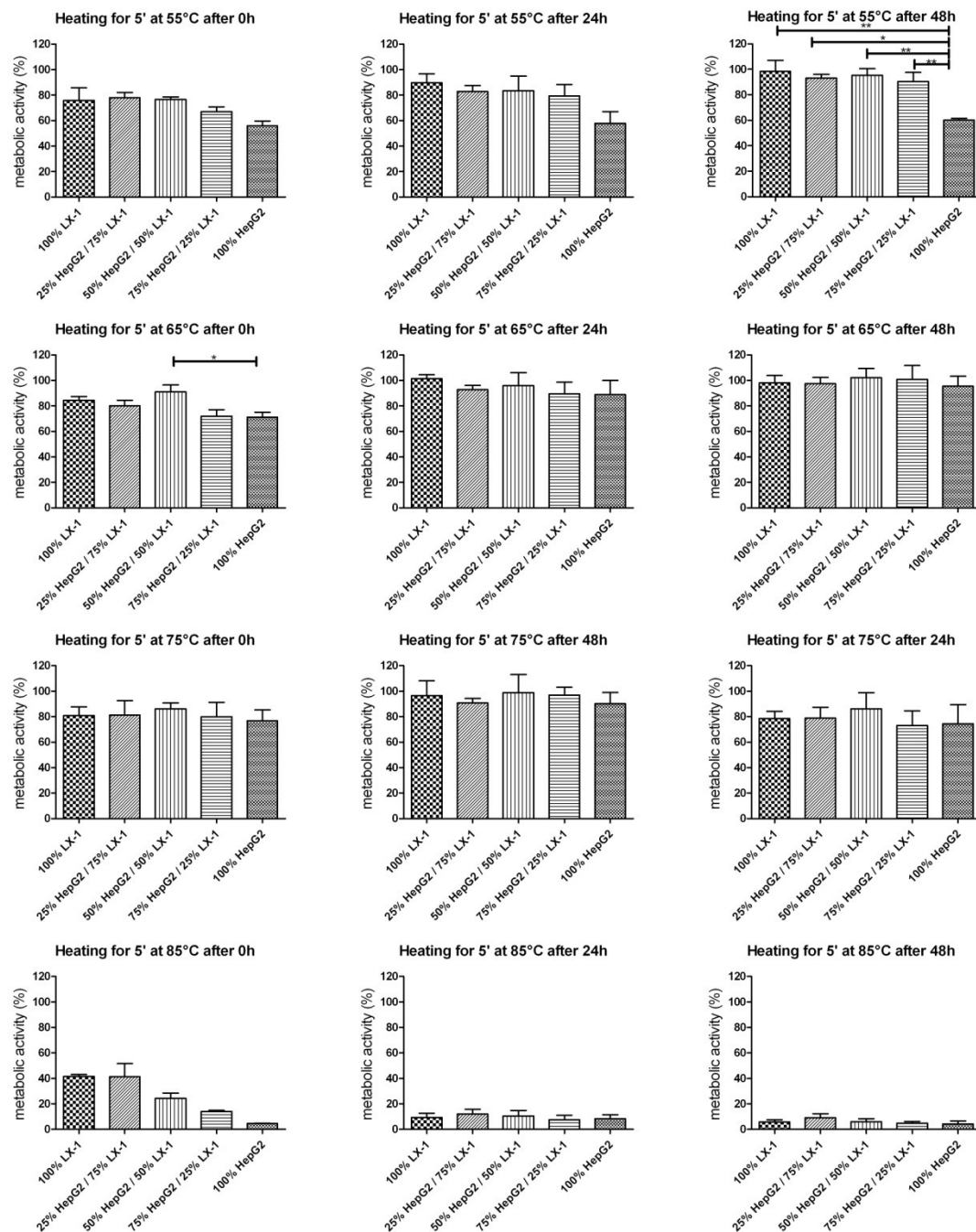


Figure 23: Heating LX-1, HepG2 cells and co-cultures for 5 minutes.

Heating HepG2 and LX-1 cells for 5 min at 55°C displays a partly significant greater heat sensitivity of HepG2 cells. Heat exposure for 5 min at 65°C and 75°C showed marginal significant differences in cell survival. Immediately after heating at 85°C the metabolic activity of HepG2 cells was lower compared to LX-1, but nearly all cells died within 24 h (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.2.2 Cell survival after temperature exposure for 10 min

Immediately and 24 h after heating (Figure 24) no significant differences could be detected. After 48 h the metabolic activity of 100% HepG2 was significant lower than 100% LX-1 ($p<0.01$), 75% HepG2/ 25% LX-1 ($p<0.05$), 50% HepG2/ 50% LX-1 ($p<0.01$) and 25% HepG2/ 75% LX-1 cells ($p<0.01$). No more significant differences could be detected after heating the cells for 10 min at 65°C and 75°C.

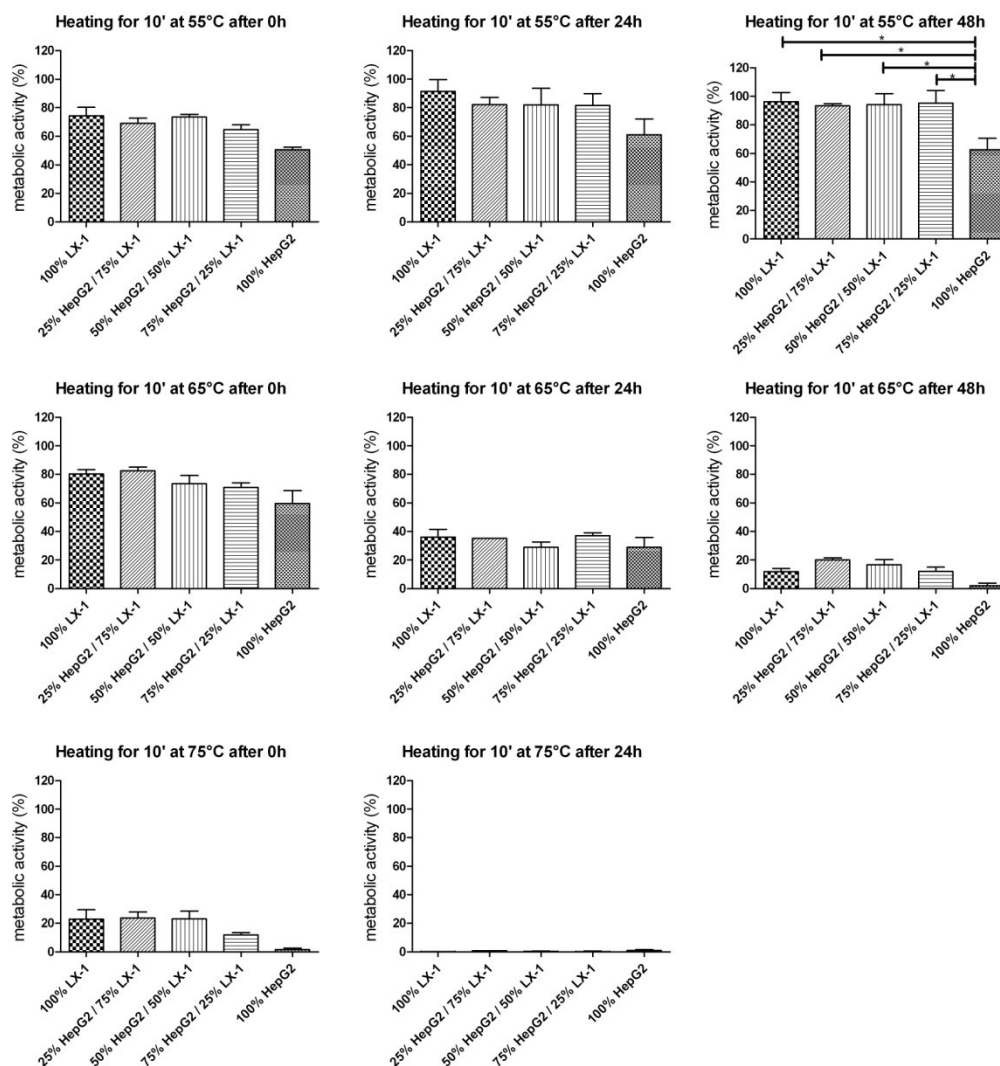


Figure 24: Heating LX-1, HepG2 cells and co-cultures for 10 minutes.

Heating HepG2 and LX-1 cells for 10 min at 55°C displays a partly significant greater heat sensitivity of HepG2 cells. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

3.2.3 Cell survival after temperature exposure for 15 minutes

Comparing 100% HepG2 to LX-1 cells (Figure 25) and their co-cultures significant differences could be detected immediately after heating as well as after 24 h of cell culture after treatment for 15 min at 55°C. The metabolic activity of 100% HepG2 was significant lower than 100% LX-1 ($p<0.001$), 50% HepG2/ 50% LX-1 ($p<0.01$) and 75% HepG2/ 25% LX-1 cells ($p<0.001$). After 24 h more viable 100% LX-1 cells ($p<0.01$), 25% HepG2/ 75% LX-1 ($p<0.05$), 50% HepG2/ 50% LX-1 ($p<0.05$) and 25% HepG2/ 75% LX-1 cells ($p<0.05$) than 100% HepG2 cells were measured.

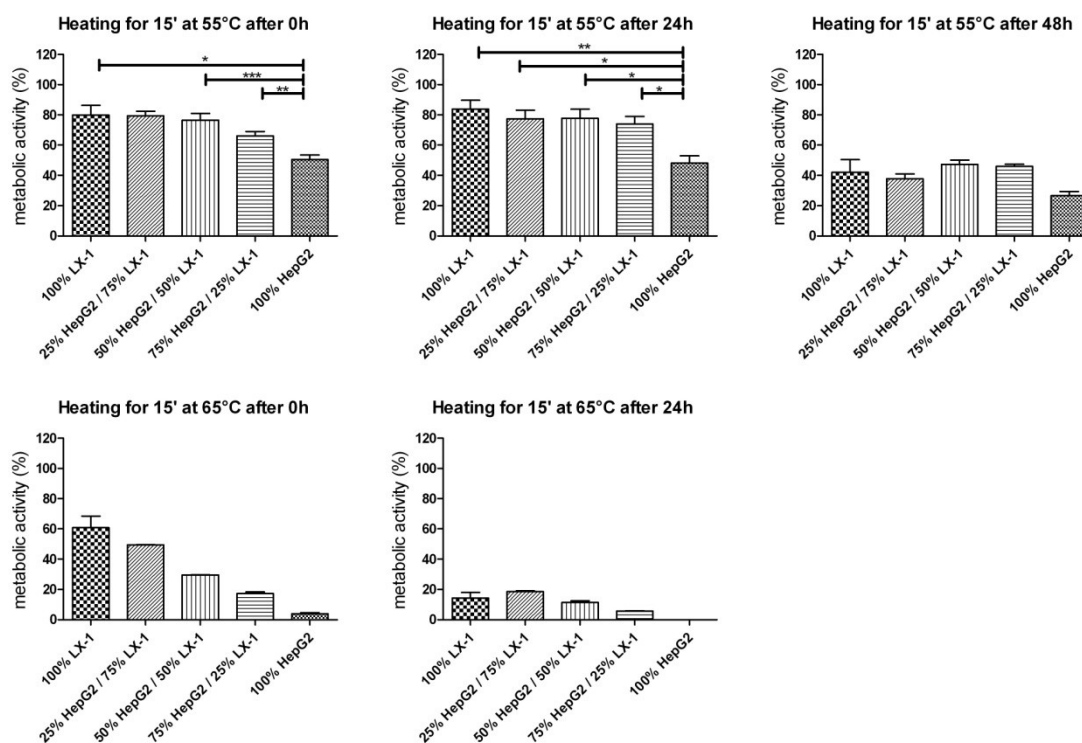


Figure 25: Heating LX-1, HepG2 cells and co-cultures after 15 minutes.

HepG2 cells showed in co-culture experiments immediately and 24 h after heating significant lower metabolic activity compared to LX-1 cells. The same results were detected immediately after heating for 10 min at 65°C (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

3.3 Comparison of MRC-5 and LX-1 cells in co-culture experiments

We wanted to create a more physiological model *in vitro* of liver fibrosis and chose the human hepatic stellate cell line LX-1 instead of MRC-5. We compared LX-1 cells to our first experiments with MRC-5 and found a very similar survival pattern.

Heating the cells at 100°C for 5 min led immediately to cell death (Figure 26) in contrast to heating at 85°C, which induces cell death over 48 h. The cells were not permanently damaged after heating at 55°C, 65°C and 75°C for 5 min.

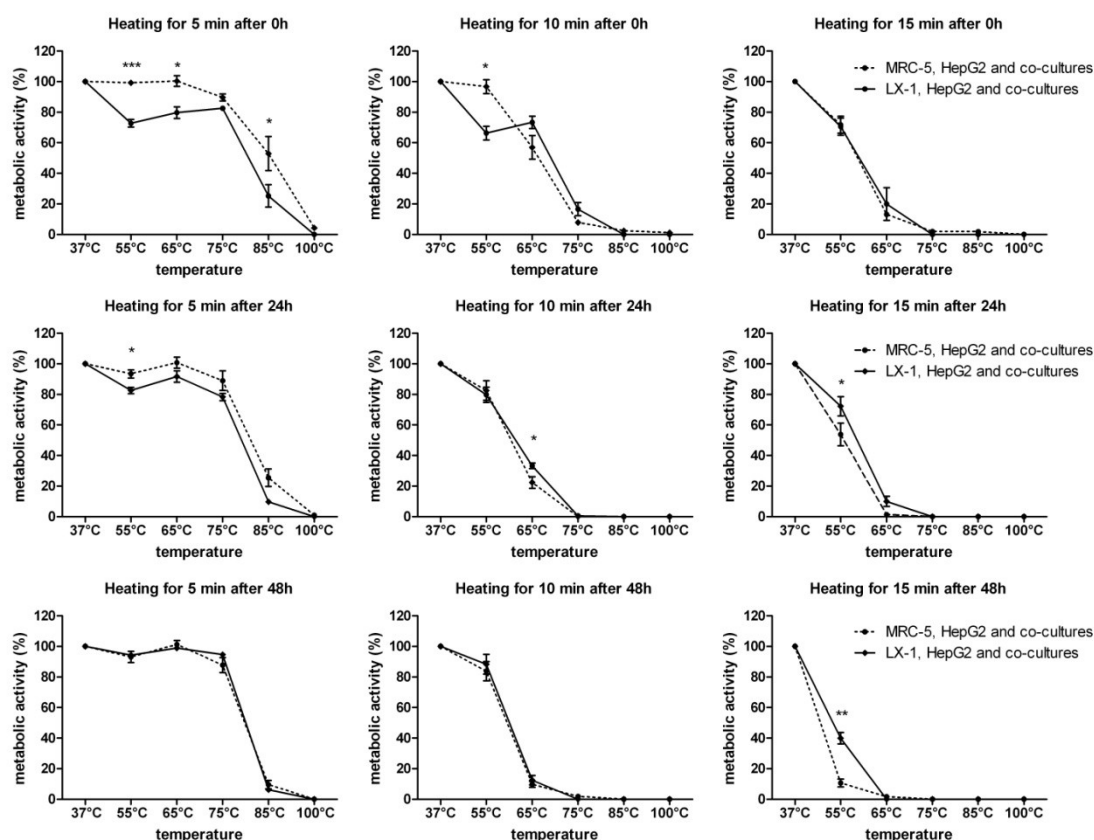


Figure 26: Shift of metabolic activity after hyperthermia ranging from 5 - 15 minutes.

Data were generated by heating the cell lines HepG2, LX-1 and their co-cultures and MRC-5, HepG2 and their co-cultures at 55°C, 65°C, 75°C and 85°C. Metabolic activity was tested either immediately after exposure to the different temperatures for different time spans (5, 10 or 15 min), after 24 h as well as after 48 h of cell culture. The data were determined using the MTS-test and normalized to the exposure at 37°C (100%). Comparison of the data was performed using one-way ANOVA. The graph displays the course of the mean values of all liver cell lines (HepG2, LX-1 and their co-cultures) over 48 h compared to previously described data of MRC-5 (and HepG2 and co-cultures). This data sets were compared via t-test and Mann-Whitney U-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The metabolic activity of MRC-5 cells and co-cultures was significantly higher immediately after heating for 5 min at 55°C ($p<0.001$), 65°C ($p<0.05$) and 85°C ($p<0.05$) than that of LX-1 and co-cultures. After 24 h the metabolic activity of MRC-5 cells and co-cultures was significantly higher after heating for 5 min at 55°C ($p<0.05$), 10 min 65°C ($p<0.05$). Heating LX-1 und HepG2 cells at 100°C, 85°C and 75°C for 10 min led immediately to cell death and kills cells heated at 65°C within 48 h. Heat exposure at 55°C did not damage the cells permanently. The metabolic activity of MRC-5 cells and co-cultures was significantly higher immediately after heating for 10 min at 55°C ($p<0.05$) than that of LX-1 and co-cultures.

Heating LX-1 und HepG2 cells at 100°C, 85°C, 75°C and 65°C for 15 min causes immediate cell death. Cells heated at 55°C showed a distinct decreasing of the metabolic activity over 48 h until only 0-25% of all cells were viable. Immediately after heating we found no significant difference. After 24 h the metabolic activity of MRC-5 and co-cultures was significantly higher after heating for 15 min at 55°C ($p<0.05$). After 48 h we could detect significantly more LX-1 and co-culture cells after heating for 15 min at 55°C ($p<0.01$).

The survival curves were nearly equal immediately after heating for 15 min, 24 h after heating for 10 min and 48 h after heating for 5 and 10 min (Figure 26).

3.4 Transwell experiments

The effect of heat exposure using different temperatures (55°C, 65°C, 75°C, 85°C) and different time spans (5 min, 10 min and 15 min) on the HCC cell line HepG2 and the HSC cell line LX-1 were determined. The cells were seeded in different numbers (25%, 50%, 75% and 100%) in different compartments and combined for heating and afterwards cultivation. The survival of LX-1 and HepG2 cells were documented immediately, 24 h and 48 h after heat treatment (Table 8 and Table 9). Figure 27 compile the shift of metabolic activity of HepG2 and LX-1 cells focusing on different temperatures.

The greatest differences in metabolic activity could be found 48 h after heating for 5 min and immediately after heating for 15 min. At these points the metabolic activity of HepG2 cells was clearly lower compared to LX-1 cells (Figure 27).

Heating HepG2 cells in transwells for 5 min showed an immediately cell death at 85°C (Figure 28) in contrast to heating at 75°C, which induced cell death over 48 h. Heat

exposure at 65°C showed a slight damage and a decrease in metabolic activity over 48 h. The cells were not permanent damaged after heating at 55°C for 5 min.

Heating HepG2 cells for 10 min showed an immediately cell death at 75°C and 65°C and killed cells heated at 65°C within 48 h. Even after heating at 55°C 50% of the cells are damaged.

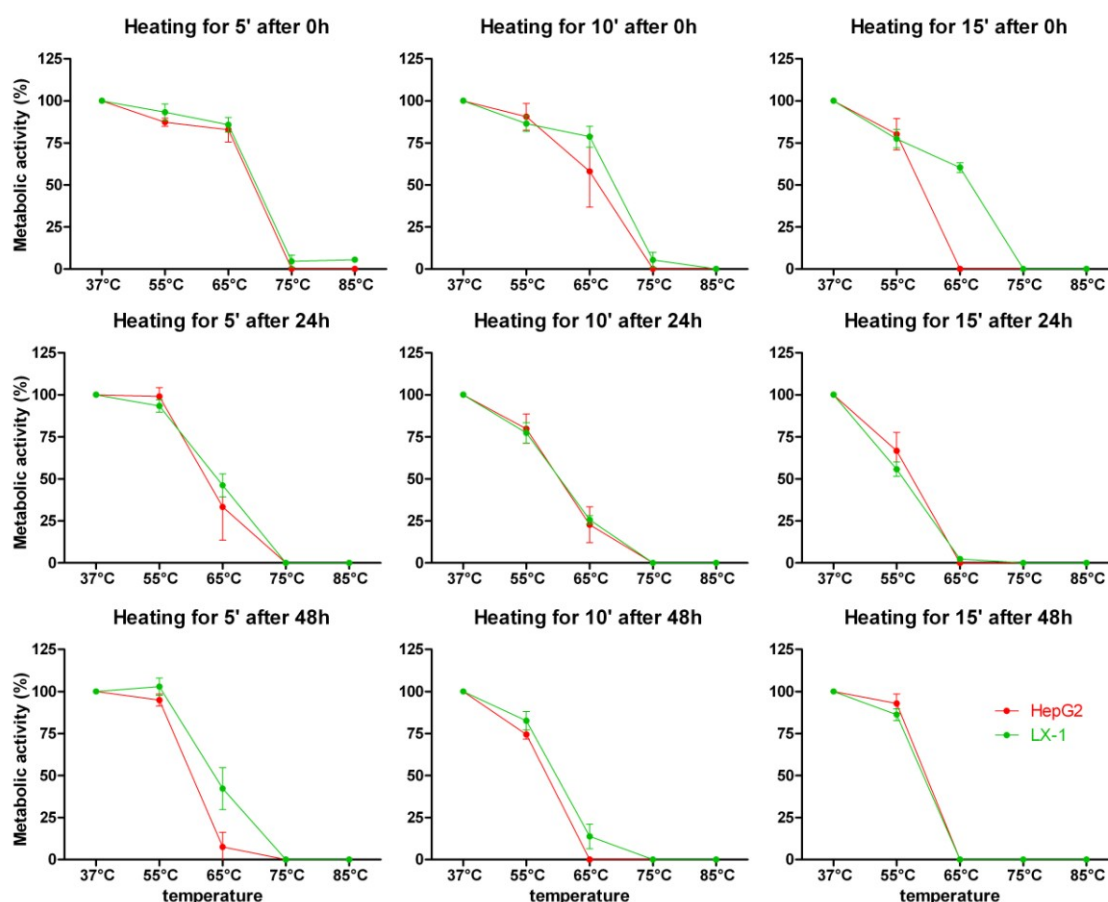


Figure 27: Heating in transwells comparing LX-1 and HepG2

Data were generated by heating the cell lines HepG2, LX-1 in transwells at 55°C, 65°C, 75°C and 85°C. Metabolic activity was tested either immediately after exposure to the different temperatures for different time spans (5, 10 or 15 minutes), after 24 h as well as after 48 h of cell culture. The data were determined using the MTS-test and normalized to the exposure at 37°C (100%). Comparison of the data was performed using one-way ANOVA.

Heating HepG2 cells at 75°C and 65°C for 15 min led also immediately to cell death and kills cells heated at 65°C within 48 h. Additionally HepG2 cells were not permanently damaged after heating at 55°C for 15 min.

Heating LX-1 cells at 85°C for 5 min in transwells caused immediate cell death (Figure 29) in contrast to heating at 75°C, which induced cell death over 48 h. Heat exposure at

65°C shows a slight damage and a decrease in metabolic activity over 48 h. The cells were not permanent damaged after heating at 55°C for 5 min.

Heating LX-1 cells at 75°C and 65°C for 10 min led immediately to cell death and kills cells heated at 65°C within 48 h. Even after heating at 55°C 50% of the cells were damaged.

Heating LX-1 cells at 75°C and 65°C for 15 min showed immediately to cell death and kills cells heated at 65°C within 48 h. Additionally, LX-1 cells were not permanently damaged after heating at 55°C for 15 min.

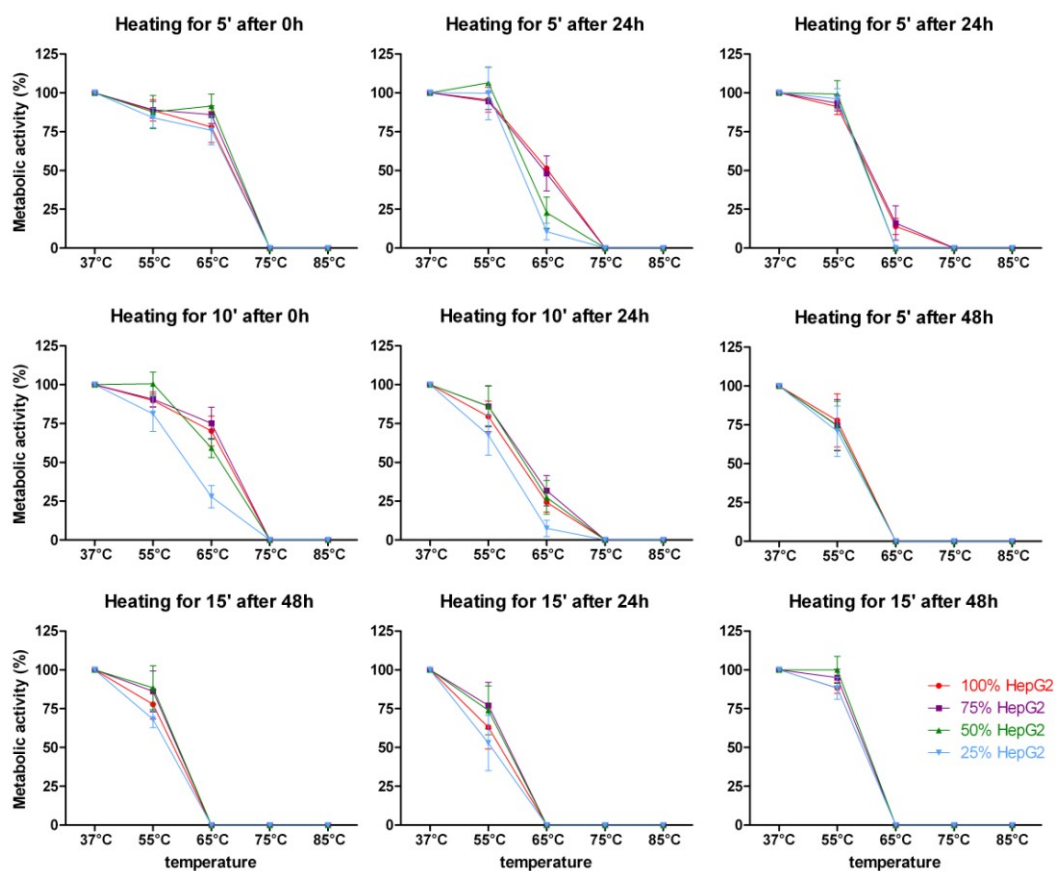


Figure 28: Shift of metabolic activity after hyperthermia ranging from 5 - 15 minutes (HepG2).

Data were generated by heating the cell lines HepG2, LX-1 in transwells at 55°C, 65°C, 75°C and 85°C. Data show heating HepG2 cells in different amounts (25-100%). Metabolic activity was tested either immediately after exposure to the different temperatures for different time spans (5, 10 or 15 minutes), after 24 h as well as after 48 h of cell culture. The data were determined using the MTS-test and normalized to the exposure at 37°C (100%). Comparison of the data was performed using one-way ANOVA.

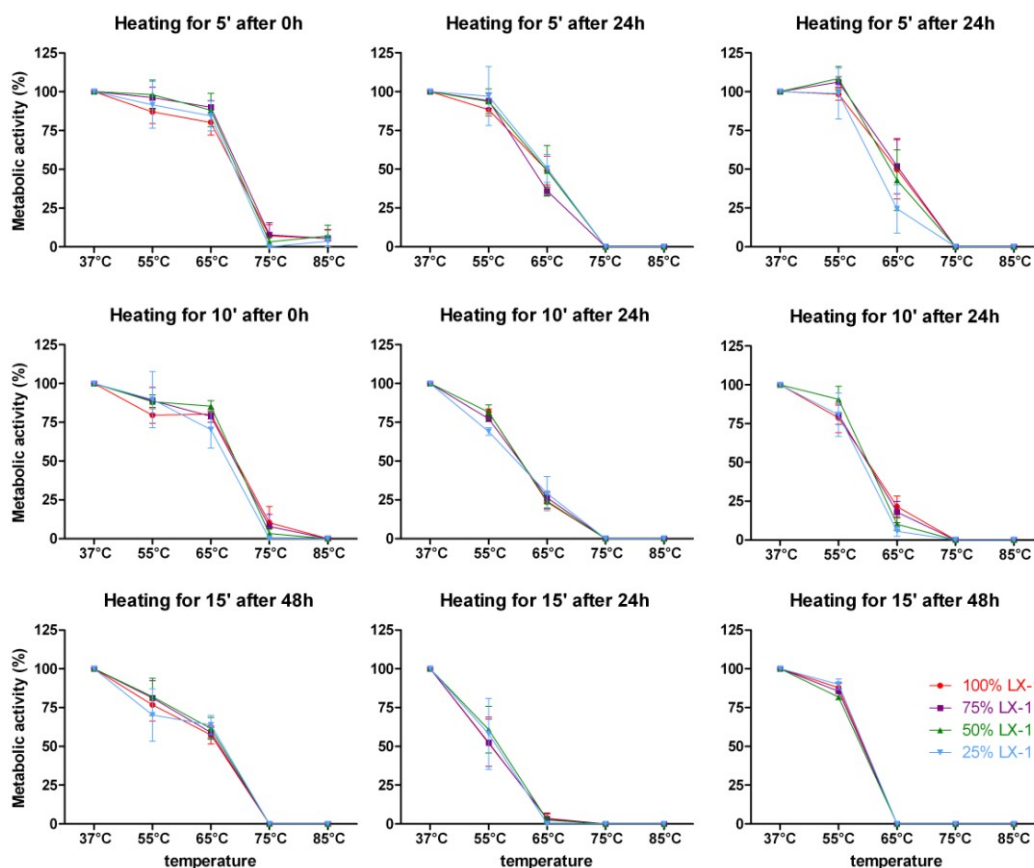


Figure 29: Shift of metabolic activity after hyperthermia ranging from 5 - 15 minutes (LX-1).

Data were generated by heating the cell lines HepG2, LX-1 in transwells at 55°C, 65°C, 75°C and 85°C. Data show heating LX-1 cells in different amounts (25-100%). Metabolic activity was tested either immediately after exposure to the different temperatures for different time spans (5, 10 or 15 minutes), after 24 h as well as after 48 h of cell culture. The data were determined using the MTS-test and normalized to the exposure at 37°C (100%). Comparison of the data was performed using one-way ANOVA.

3.4.1 Cell survival after temperature exposure for 5 minutes

Heating LX-1 and HepG2 cells at 55°C in different compartments of transwells showed no significant differences in metabolic activity (Figure 30; Table 8 and Table 9). Additionally no remaining cell damage could be detected.

Heat exposure at 65°C for 5 minutes led to death of nearly all HepG2 cells and of about 50% of LX-1 cells over 48 h. If HepG2 cells are cultivated with no or a small numbers (25%) of LX-1 cells, these cells showed a better metabolic activity compared to cultivation with a higher LX-1 cell concentration (50% and 75%).

Immediately after heating at 75°C and 85°C a small numbers of LX-1 cells survived, but they died within 24 h.

Table 8: Results of transwell experiments (HepG2).

Data were generated by heating the cell lines HepG2 (and LX-1) at 55°C, 65°C, 75°C and 85°C in transwells. The table shows only metabolic activity of HepG2 cells, which was tested either immediately after exposure to the different temperatures for different time spans (5, 10 or 15 minutes), after 24 h as well as after 48 h of cell culture. The data were determined using the MTS-test and normalized to the exposure at 37°C (100%). The table shows the percentage of metabolic activity (first column) and the standard error (second column) after heating. 5' 0 h: viability measurement immediately after exposure to the different temperatures for 5 minutes; 5' 24 h viability measurement after 24 h, etc. The average of cell viability and the standard error were calculated from 5 repeated tests.

	mean	SEM	mean	SEM	mean	SEM	mean	SEM
5' 0h	100% HepG2		75% HepG2		50% HepG2		25% HepG2	
55°C	88,69	6,87	89,06	5,51	87,70	10,62	83,86	6,32
65°C	78,01	9,89	85,93	5,63	91,47	7,61	75,85	9,19
75°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
85°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
10' 0h	100% HepG2		75% HepG2		50% HepG2		25% HepG2	
55°C	89,78	4,20	90,54	4,81	100,60	7,63	81,21	11,36
65°C	70,12	9,67	75,20	10,22	59,19	6,09	27,84	7,22
75°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
15' 0h	100% HepG2		75% HepG2		50% HepG2		25% HepG2	
55°C	77,80	9,51	86,17	13,15	88,52	14,11	68,11	5,34
65°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
5' 24 h	100% HepG2		75% HepG2		50% HepG2		25% HepG2	
55°C	95,50	8,10	94,64	5,30	106,40	10,06	99,70	17,08
65°C	51,28	0,37	48,20	11,25	22,91	10,01	10,67	5,27
75°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
85°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
10' 24 h	100% HepG2		75% HepG2		50% HepG2		25% HepG2	
55°C	79,56	9,83	86,09	13,23	86,23	12,78	67,67	13,01
65°C	24,27	6,29	31,79	9,72	27,47	10,82	7,41	5,13
75°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
15' 24 h	100% HepG2		75% HepG2		50% HepG2		25% HepG2	
55°C	63,08	13,97	76,98	15,07	73,93	15,72	52,85	17,78
65°C	24,27	6,29	31,79	9,72	27,47	10,82	7,41	5,13
5' 48 h	100% HepG2		75% HepG2		50% HepG2		25% HepG2	
55°C	91,01	5,14	93,41	4,89	99,16	8,62	96,17	6,55
65°C	14,00	5,17	16,21	11,05	0,00	0,00	0,00	0,00
75°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
85°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
10' 48 h	100% HepG2		75% HepG2		50% HepG2		25% HepG2	
55°C	77,80	17,00	74,77	16,55	74,39	15,81	70,89	16,21
65°C	14,00	5,17	16,21	11,05	0,00	0,00	0,00	0,00
75°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
15' 48 h	100% HepG2		75% HepG2		50% HepG2		25% HepG2	
55°C	88,33	2,56	94,96	3,83	99,92	6,16	88,22	5,07
65°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00

Table 9: Results of transwell experiments (LX-1)

Data were generated by heating the cell lines LX-1 (and HepG2) at 55°C, 65°C, 75°C and 85°C in transwells. The table shows only metabolic activity of LX-1 cells, which was tested either immediately after exposure to the different temperatures for different time spans (5, 10 or 15 minutes), after 24 h as well as after 48 h of cell culture. The data were determined using the MTS-test and normalized to the exposure at 37°C (100%). The table shows the percentage of metabolic activity (first column) and the standard error (second column) after heating. 5' 0 h: viability measurement immediately after exposure to the different temperatures for 5 minutes; 5' 24 h viability measurement after 24 h, etc. The average of cell viability and the standard error were calculated from 5 repeated tests.

	mean	SEM	mean	SEM	mean	SEM	mean	SEM
5' 0h	100% LX-1		75% LX-1		50% LX-1		25% LX-1	
55°C	86,98	7,55	96,22	6,78	98,19	9,25	91,54	15,07
65°C	80,29	8,31	90,03	4,19	88,21	10,80	84,44	9,55
75°C	7,19	7,19	7,82	7,82	3,29	3,29	0,00	0,00
85°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
10' 0h	100% LX-1		75% LX-1		50% LX-1		25% LX-1	
55°C	79,55	5,23	88,88	8,59	88,21	4,44	89,59	18,16
65°C	80,28	3,09	78,75	3,69	85,33	3,71	70,27	11,98
75°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
15' 0h	100% LX-1		75% LX-1		50% LX-1		25% LX-1	
55°C	76,67	10,39	81,18	11,21	81,99	12,00	70,11	16,79
65°C	57,39	5,78	58,92	3,36	61,55	7,02	63,77	6,16
5' 24 h	100% LX-1		75% LX-1		50% LX-1		25% LX-1	
55°C	88,34	4,06	94,27	4,95	93,67	8,00	97,09	18,98
65°C	49,12	9,25	35,99	2,63	49,01	16,27	50,48	8,92
75°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
85°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
10' 24 h	100% LX-1		75% LX-1		50% LX-1		25% LX-1	
55°C	81,66	2,00	77,26	1,94	81,65	4,53	69,00	2,41
65°C	23,53	4,52	26,26	3,62	23,97	4,30	29,01	11,07
75°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
15' 24 h	100% LX-1		75% LX-1		50% LX-1		25% LX-1	
55°C	52,01	16,87	52,46	15,18	60,75	15,00	58,03	22,84
65°C	3,64	3,64	3,18	3,18	2,40	2,40	0,00	0,00
5' 48 h	100% LX-1		75% LX-1		50% LX-1		25% LX-1	
55°C	98,33	3,76	106,20	3,33	108,50	7,54	98,78	16,40
65°C	49,86	19,19	51,94	17,80	42,83	19,60	24,41	15,64
75°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
85°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
10' 48 h	100% LX-1		75% LX-1		50% LX-1		25% LX-1	
55°C	78,65	9,47	80,67	6,26	90,67	8,46	80,70	13,98
65°C	21,54	6,75	17,95	6,88	10,16	4,08	5,58	3,23
75°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
15' 48 h	100% LX-1		75% LX-1		50% LX-1		25% LX-1	
55°C	87,82	1,90	85,53	3,90	81,74	1,21	89,96	2,46
65°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00

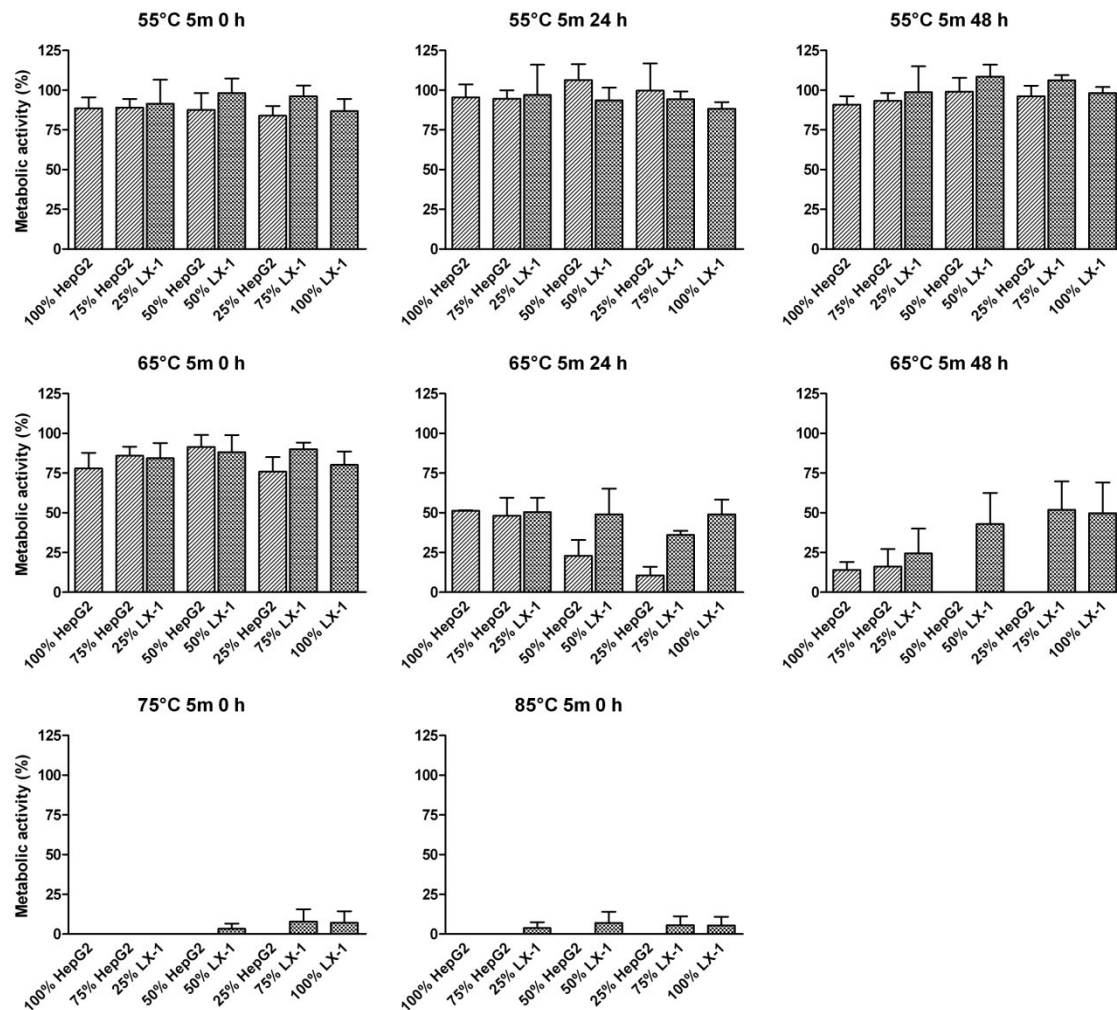


Figure 30: Heating HepG2 and LX-1 cells for 5 min in transwells.

Immediately and up to 48 h after heating at 55°C no significant differences could be found measuring metabolic activity via MTS test. Heating at 65°C showed that the lower the concentration of HepG2 cells in the transwells was the lower also was the metabolic activity of the cells. Possibly the small amount of seeded HepG2 cells (only $2,5 \times 10^4$ at 25% HepG2) is comparatively more heat sensitive than a higher amount (e.g. 100% HepG2, which equates 10×10^4 cells) of cells. Immediately after heating at 75°C and 85°C for 5 min a survival of a small amount of LX-1 cells could be measured, but these cells also died within 24 h (data not shown).

3.4.2 Cell survival after temperature exposure for 10 minutes

Heating LX-1 and HepG2 cells at 55°C for 10 min showed no significant differences in metabolic activity (Figure 31; Table 8 and Table 9). Immediately after heating at 65°C for 10 min metabolic activity of 25% HepG2 cells was significant lower ($p < 0.05$) compared to LX-1 cells. Over 48 h all HepG2 cells died.

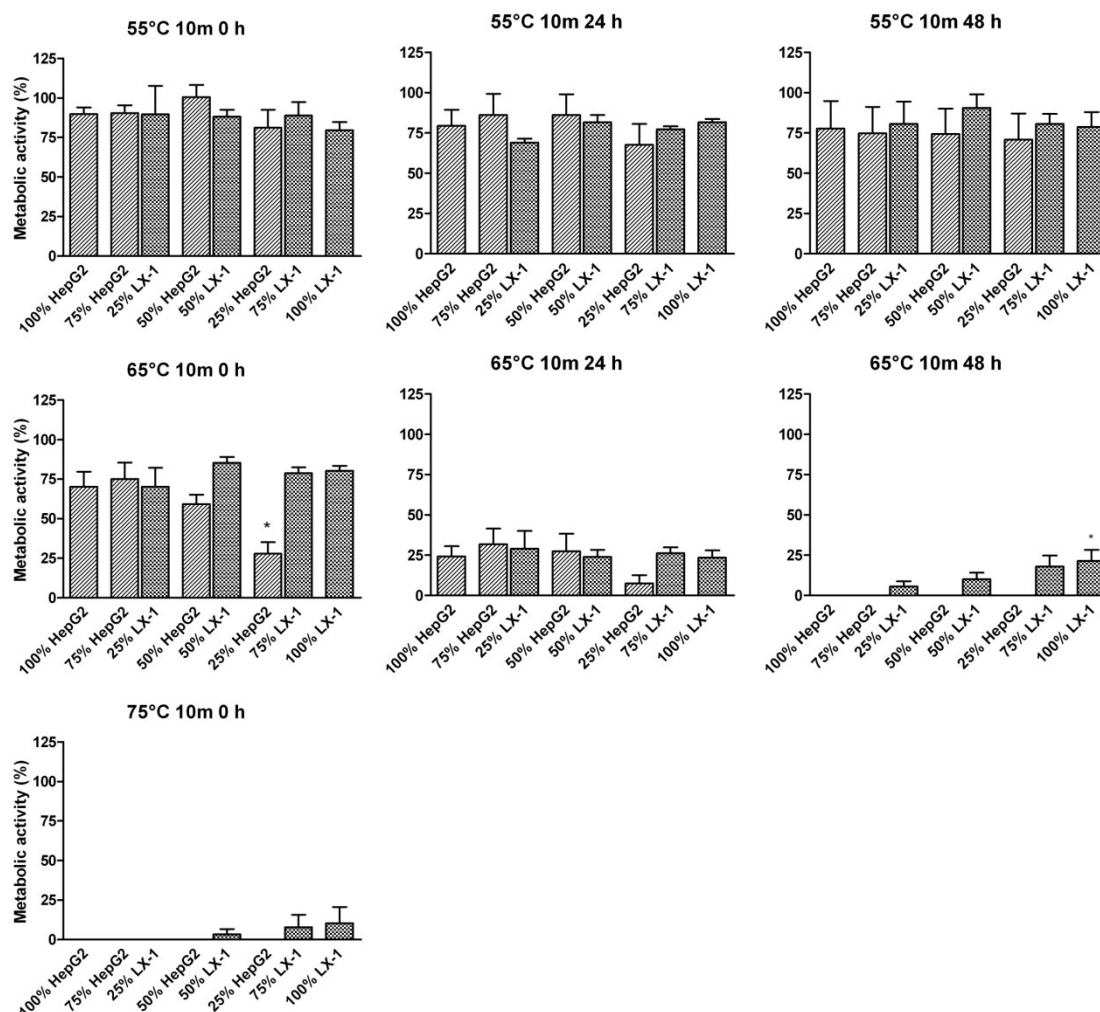


Figure 31: Heating HepG2 and LX-1 cells for 10 min in transwells.

Immediately and up to 48 h after heating at 55°C no significant differences could be found when measuring metabolic activity via MTS test. Heating at 65°C showed that the lower the concentration of HepG2 cells was in the transwells the lower also was the metabolic activity of the cells. Immediately after heating at 75°C for 5 min a survival of a marginal amount of LX-1 cells could be measured, but these cells also died within 24 h (data not shown).

3.4.3 Cell survival after temperature exposure for 15 minutes

Heating LX-1 and HepG2 cells at 55°C for 15 min showed no significant differences in metabolic activity (Figure 32; Table 8 and Table 9). After heating at 65°C for 15 min killed immediately all HepG2 cells ($p < 0.001$), but LX-1 cells died within 24 h, too.

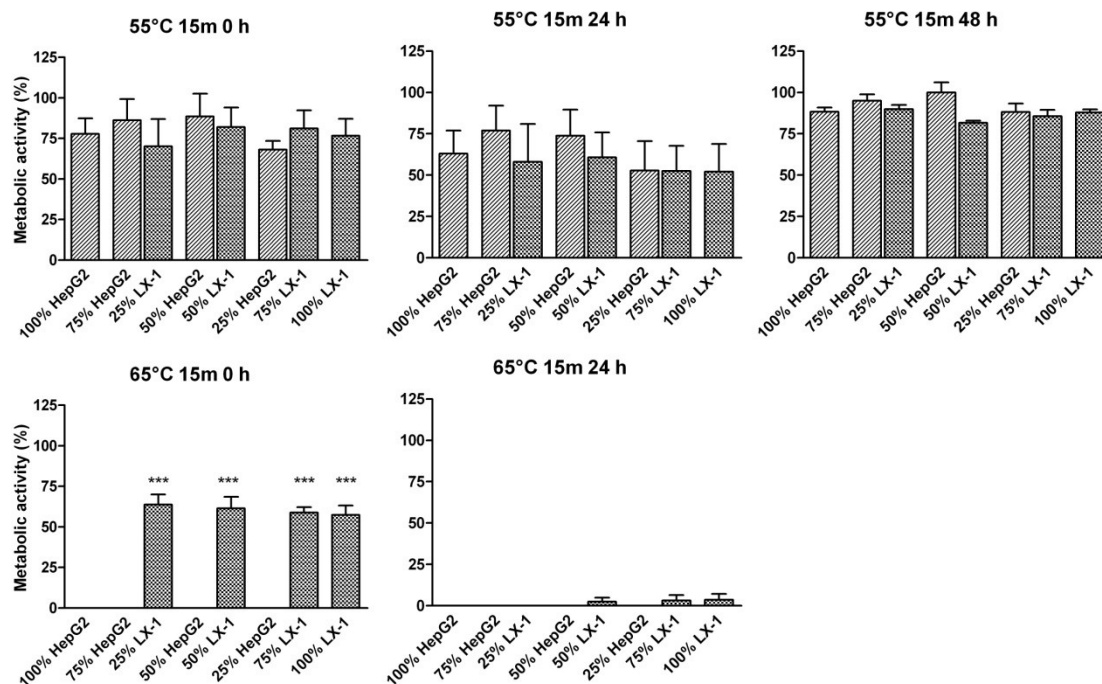


Figure 32: Heating HepG2 and LX-1 cells for 15min in transwells.

Immediately and up to 48 h after heating at 55°C no significant differences could be found measuring metabolic activity via MTS test. Heating at 65°C showed that the lower the concentration of HepG2 cells in the transwells the lower the metabolic activity of the cells.

3.5 Comparison of co-culture and transwell experiments

In the co-culture model HepG2 and LX-1 cells were seeded in different compositions together in one well and we measured a common metabolic activity. In contrast cells in the transwell model were seeded separated in two different compartments and were combined for heating and afterwards cultivation. We measured the metabolic activity for each cell line individually.

Comparing co-culture and transwell experiments, we detected very different rates of cell survival. Generally, cells cultivated together in one well seem to be more heat resistant compared to transwells (Figure 33), except at a temperature of 55°C (for 15min). This time point shows after 48 h a highly significant different survival rate in transwells then in co-cultures ($p < 0.001$).

Immediately after heating at 65°C for 5 min we found no differences. After 24 and 48 h the metabolic activity in the co-culture model was significantly higher compared to the transwell model ($p < 0.01$ to $p < 0.001$).

Immediately after heat exposure for 10 min significant differences between the two experiments were detected ($p<0.001$) as well as for 15 min ($p<0.01$ to $p<0.001$). The metabolic activity in the co-culture model stayed constant in contrast to the transwell model, where the metabolic activity decreased within 24 and 48 h. Heating for 10 and 15 min at 65°C showed a common decrease to 0-25% over 48 h.

After heating for 5 min at 75°C highly significant differences between co-culture and transwell results were observed immediately and over 48 h of cultivation ($p<0.001$). Cells heated in transwells died immediately in contrast to cells of co-culture experiments, where they showed a constant viability of 75-80%. Heating at 75°C for 10 min and 85°C for 5 min showed immediately and over 48 h a small co-culture survival (up to 25%) compared to transwell experiments, where no living cells were detected.

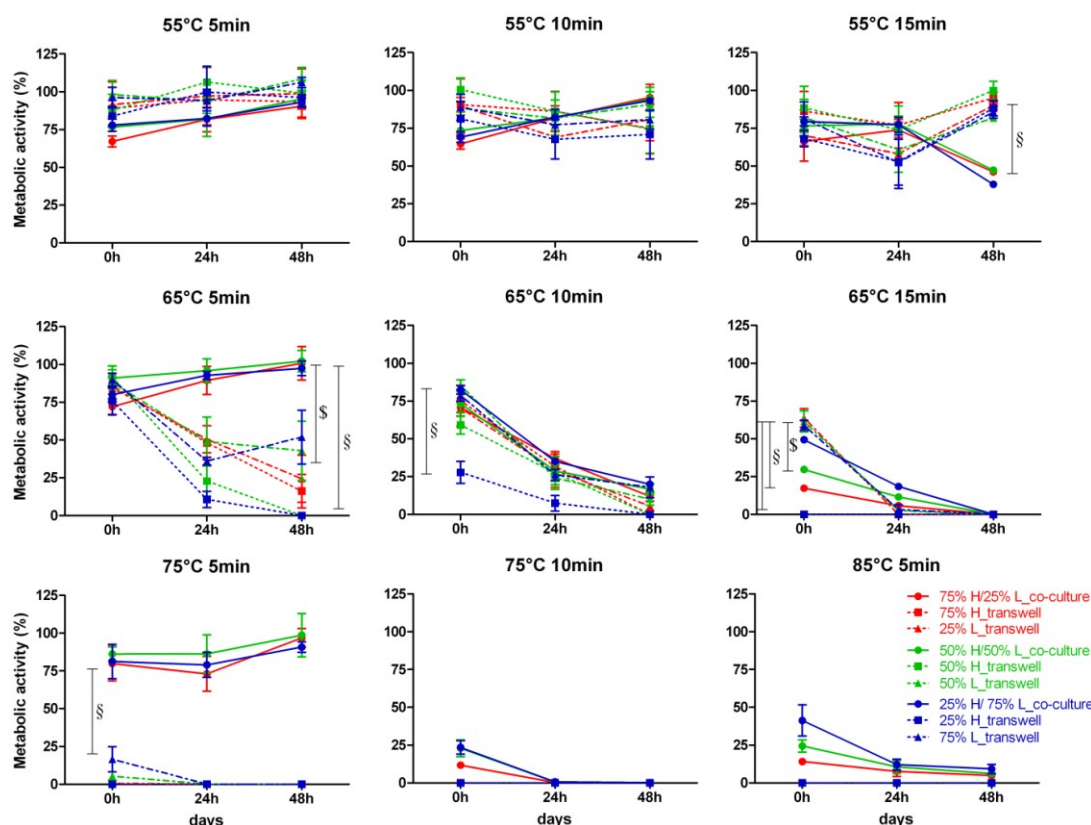


Figure 33: Comparing co-culture and transwell heating experiments.

Cultivating HepG2 (H) and LX-1 (L) cells in co-cultures led to a greater cell survival than cultivating them in transwells (* $p<0.05$, \$ $p<0.01$, § $p<0.001$).

3.6 Flow Cytometry

Flow Cytometry was performed after heating to distinguish between apoptosis and necrosis. Prior to heating, the cell lines were stained intracellular for better distinction during measurement (Figure 35). After heating the cells were stained with a viability dye (eFluor 450) and Annexin V (stains phosphatidylserine) and then measured using an LSR II Flow Cytometer.

During apoptosis there is a time lag between externalization of phosphatidylserine (PS) and eFluor 450 uptake in contrast to necrosis. So during PS externalization the cell population shifts from lower left quadrant (eFluor 450 and Annexin V negative) to the lower right quadrant (eFluor 450 negative, Annexin V positive). Thereafter, the membrane loses the membrane integrity and eFluor 450 is able to translocate into the cells, which leads to a shift of the population to the upper right quadrant (eFluor 450 positive, Annexin V positive; Figure 34). During necrosis PS translocation coincides with the permeabilization of the cell membrane and the population would shift from lower left quadrant immediately to the upper right quadrant [121]. All investigated time points (55°C 15min, 65°C 10 min, 65°C 15 min and 75°C 5 min) showed without exception apoptotic cell death (Figure 34).

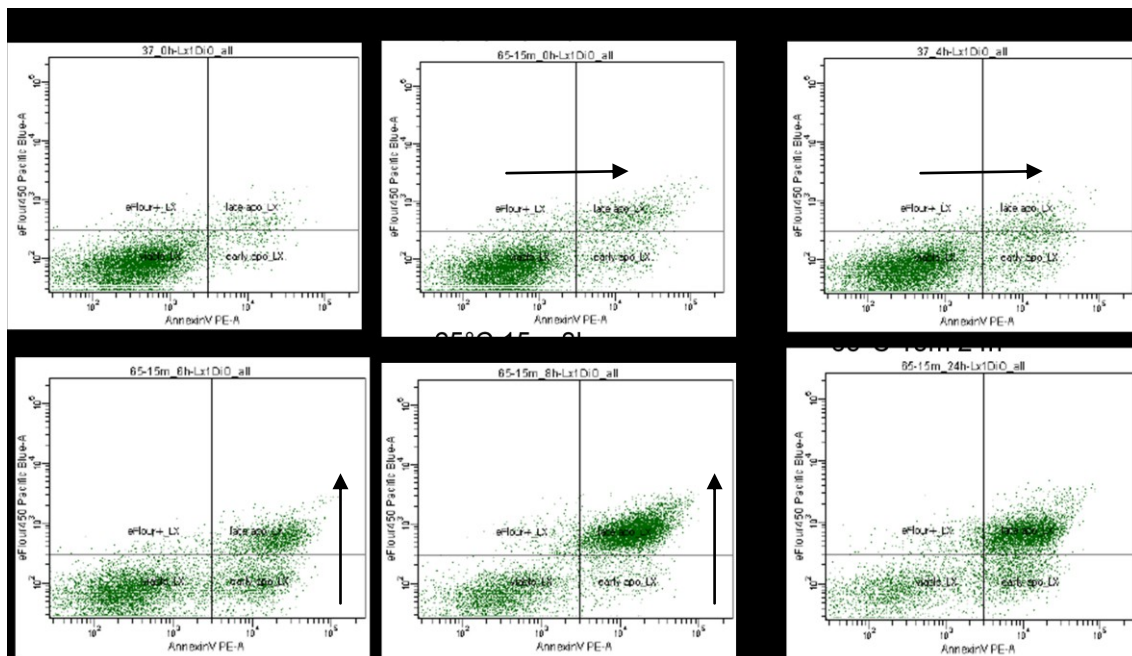


Figure 34: Apoptotic cell death after heating at 65°C for 15 min (Flow Cytometry).

Cells were analysed by Flow Cytometry for externalization of phosphatidylserine (binding of Annexin V) and for loss of membrane integrity (eFluor 450 uptake). This leads to the conclusion that these cells were dying apoptotic not necrotic.

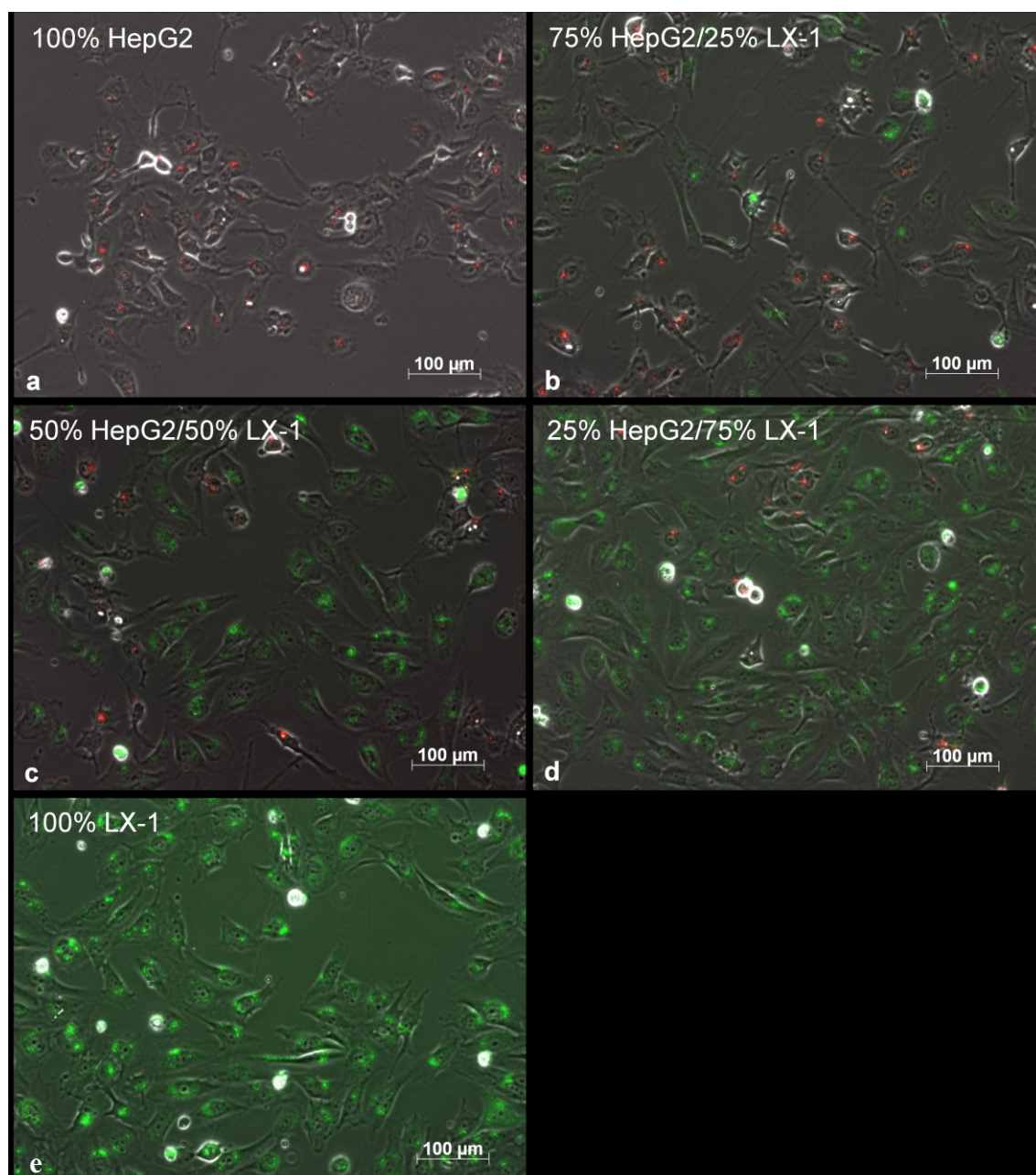


Figure 35: Intracellular staining of HepG2 and LX-1 cells in co-culture.

HepG2 (red, DiD), LX-1 (green, DiO) and their co-cultures of the 37°C control before flow cytometry measurement.

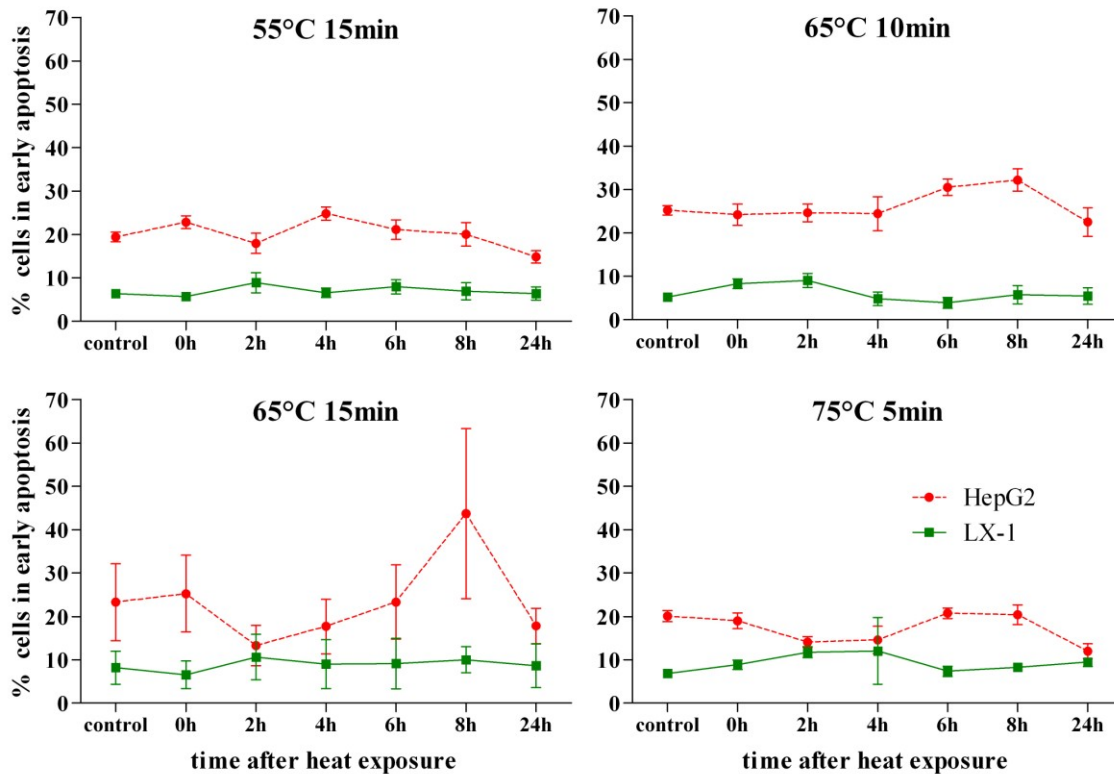


Figure 36: Percentage of cells in early apoptosis after heating (Flow Cytometry).

Early apoptotic cells are defined as Annexin V positive and eFluor 450 negative cells. They were compared to untreated and unstained controls.

Evaluation of Flow Cytometry data showed a constant numbers of cells in early apoptosis (eFluor 450 negative, Annexin V positive, Figure 36). Over 24 h approximately 20-30% of HepG2 cells were identified as early apoptotic. An unusual peak of about 40% with a high standard error (20%) was detected 8 h after heating at 65°C at 15 min. The numbers of LX-1 cells in early apoptosis was constant between 5 and 15% subsequent heating.

After heat exposure at 55°C for 15 min we found only a few (~5%) late apoptotic cells (eFluor 450 positive and Annexin V positive; Figure 37). Eight hours after heating at 65°C for 10 min a rise to 30% of HepG2 cells in late apoptosis could be displayed and led to a peak of 65% after 24 h. LX-1 cells showed only after 24 h an increase to 30% late apoptotic cells. Immediately after heating a marginally increased numbers of late apoptotic LX-1 (~10%) and HepG2 cells (~20%) was found. This peaked in 55-60% (8 h and 24 h) of late stage apoptotic LX-1 cells and 25% (8 h) and 50% (24 h) of HepG2 cells. Following heat exposure to 75°C for 5 min we found a constantly low level of LX-1 cells (5-10%) in late apoptosis that increased severely to 35% after 24 h.

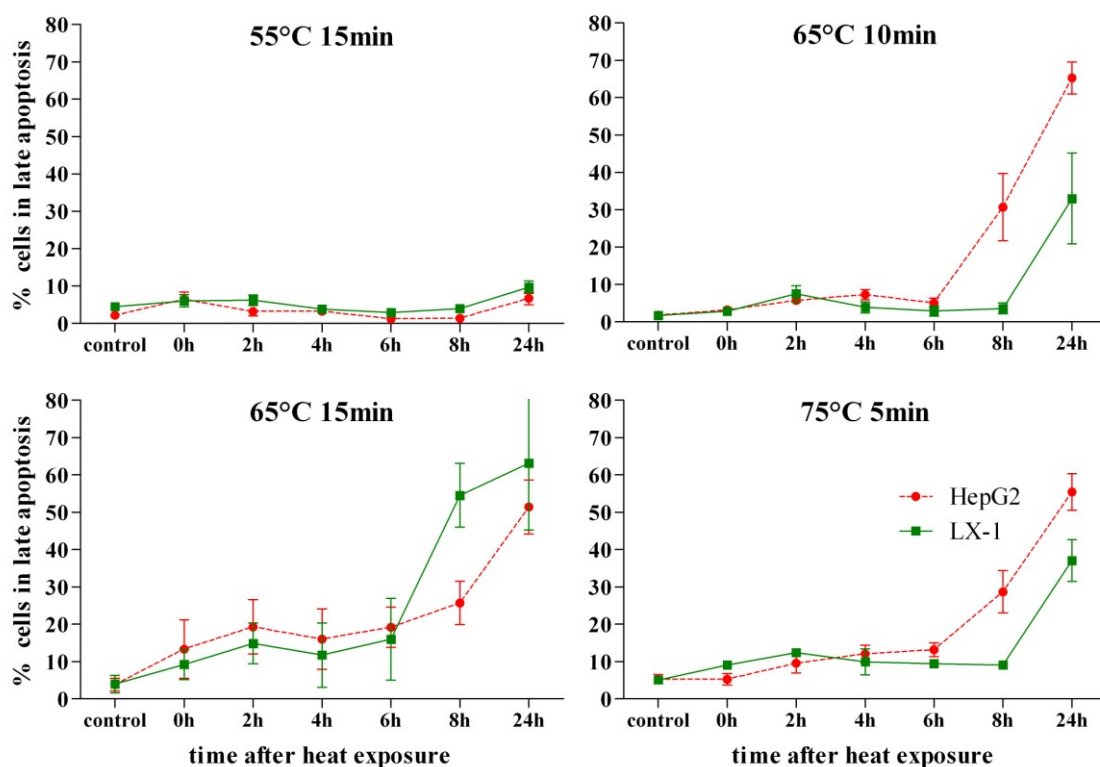


Figure 37: Percentage of cells in late apoptosis after heating (Flow Cytometry).

Late apoptotic cells are defined as Annexin V positive and eFluor 450 positive cells. They were compared to untreated and unstained controls.

In contrast to LX-1, HepG2 cells showed a constantly increasing amount of late apoptotic cells (5 to 15%) within 6 h and then a severe rise to 30% after 8 h and 55% after 24 h.

In comparison to early and late apoptotic cells the total amount of viable cells (Figure 38) after heating decreased clearly after 8 and 24 h (except at 55°C). Flow Cytometry showed that LX-1 cells were more heat resistant compared to HepG2 cells.

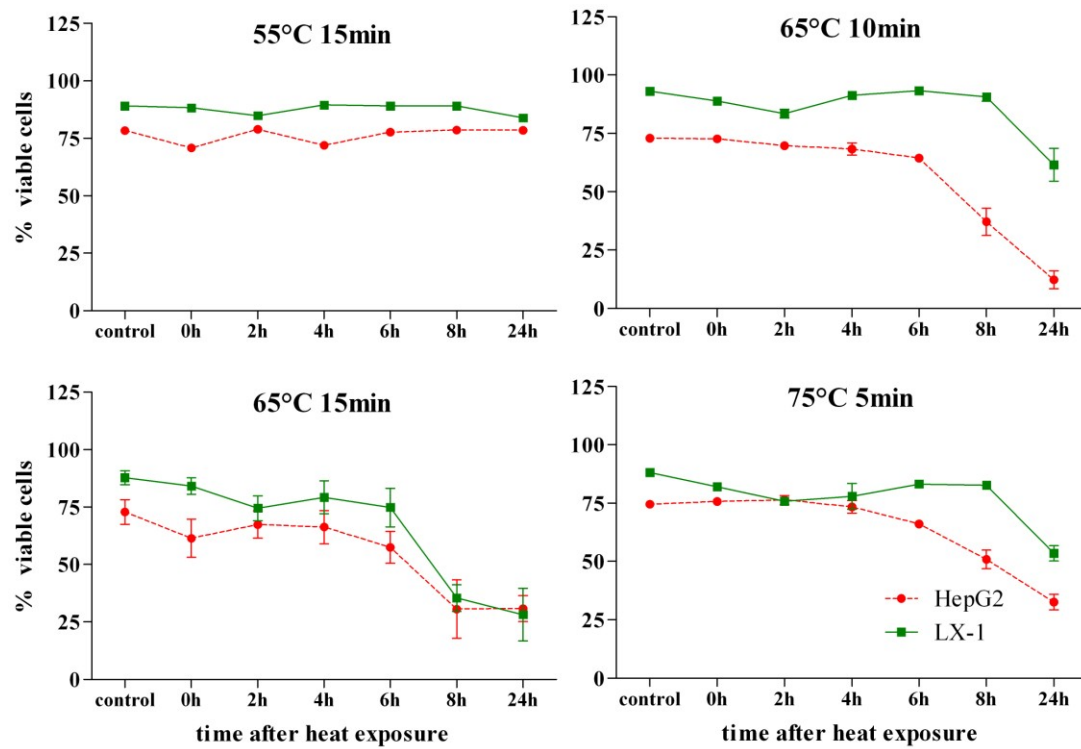


Figure 38: Percentage of viable cells after heating (Flow Cytometry).

Viable cells are defined as Annexin V and eFluor 450 negative cells. They were compared to untreated and unstained controls.

3.7 Animal experiments

We investigated the wound healing process in the liver of healthy pigs after radiofrequency ablation (RFA). Two lesions were performed in each liver, the first one near a vessel (central lesion) and second one far away (peripheral lesion). After RFA treatment the pigs (n=2) lived for different live spans (0d, 1d, 2d, 1w, 2w, 1m, 2m, 3m) till they were euthanized and the liver was taken. One of the two treated porcine livers was formalin fixated by perfusion fixation and used for large scale blocks.

These blocks were sliced and stained for MB and CAB (Figure 40). The standard paraffin blocks were stained for CAB (Figure 39). Additionally we used Caspase 3 (apoptosis), Hsp70 (heat stress), Ki67 (proliferation), Calprotectin (marker for macrophages and monocytes), GFAP (marker for activated hepatic stellate cells) and α -SMA (marker for myofibroblasts) for further characterization of the transition zone.

3.7.1 Overview

Immediately after RFA the area showed a good limited lesion with carbonisation in the middle and bleeding in the range of the border to the healthy tissue (Figure 39a, Figure 40a). Within the first two day the bleeding (red rim) became stronger and the visual lesion in the CT increased in size (Figure 39b,c, Figure 40b,c). After one week the lesion developed an α -SMA fibrotic capsule (blue rim), still followed by a red rim containing erythrocytes (Figure 39d, Figure 40d). After two weeks the bleeding disappeared and the fibrotic rim became bigger (Figure 39e, Figure 40e). After one month the fibrotic capsule shrank and the carbonization was replaced by connecting tissue (Figure 39f, Figure 40f). After another month the lesion was still decreasing and after 3 month the capsule was not detectable only in one pig (Figure 39g, h, Figure 40g).

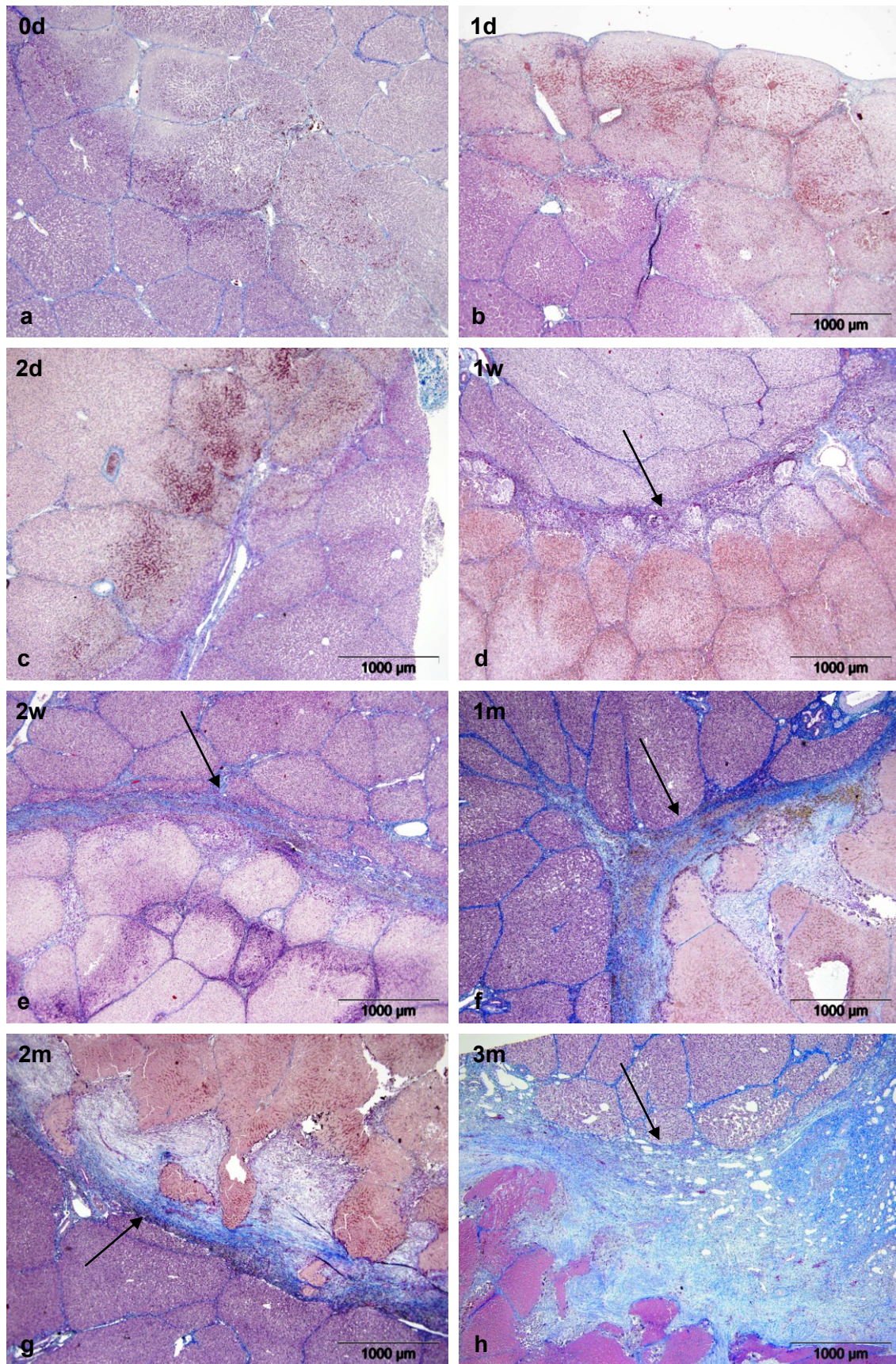


Figure 39: CAB staining of survivor pigs (immediately after RFA up to 3 months).

L=RFA lesion; arrow shows the development of the fibrotic capsule.

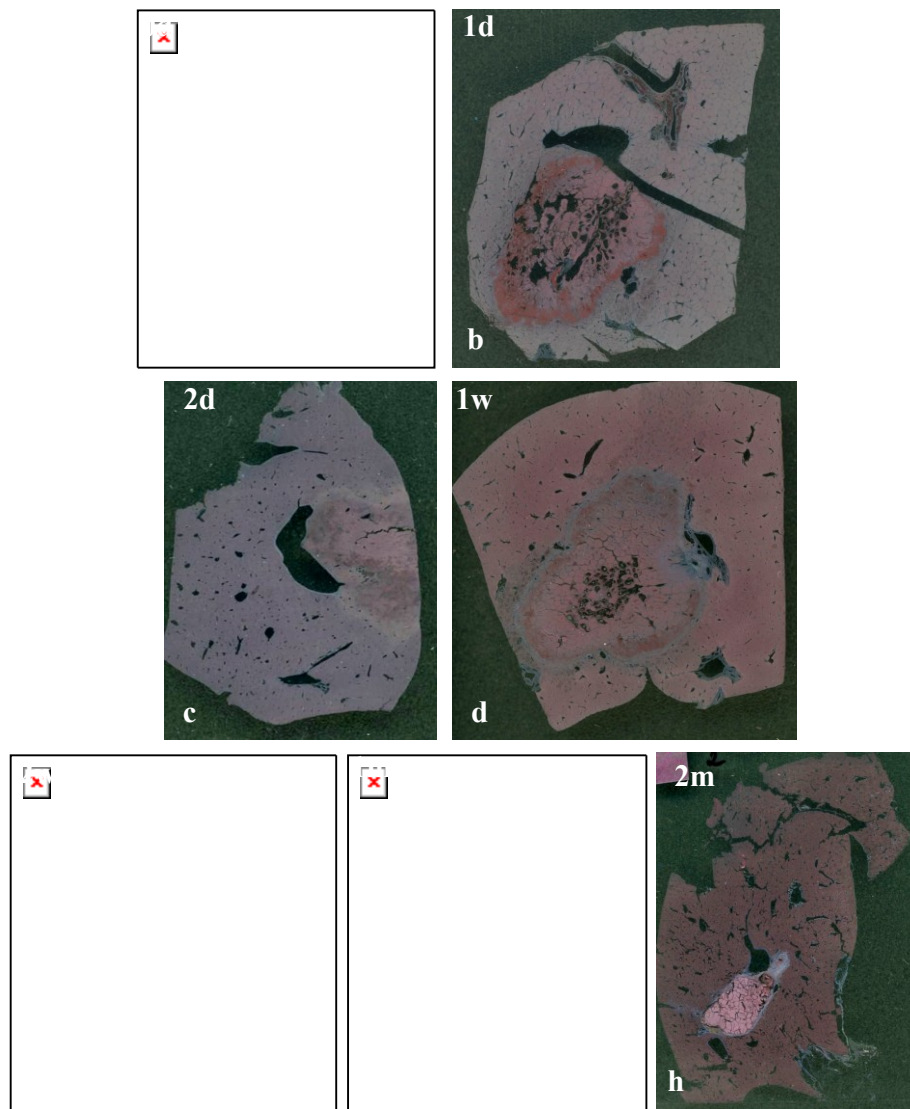


Figure 40: CAB staining of Survivor pigs.

Healthy pigs were treated via RFA and euthanized at different time spans. CAB staining shows development of the ablation zone surrounding tissue over 2 month (d = days, w = weeks, m = months).

3.7.2 Immunohistochemical staining

Immediately (0d) after RFA cells of the transition zone are stained for Ki-67, Calprotectin and Caspase 3. After one day (1d) we found still proliferating as well as apoptotic cells in the ablation area. Myofibroblasts were migrating from the vessels into the transition zone. These fibroblasts were Hsp70 positive just as heat damaged hepatocytes outside the lesion. Furthermore we could detect strong Caspase 3 and Hsp70 positive cells in the transition zone (Figure 42). Two days (2d) after RFA staining pattern was equal to the previous day.

Table 10: Antibody staining in transition zone after RFA

<i>after RFA</i>	<i>Caspase 3</i>	<i>Ki-67</i>	<i>Hsp70</i>	<i>Calprotectin</i>	<i>GFAP</i>	<i>α-SMA</i>
0 d	+	+	-	++	-	-
1 d	++	++	+++	++	-	+++
2 d	++	++	+++	+	+	+++
1 wo	-	++	-	+	-	+++
2 wo	-	++	-	+	-	+++
1 mo	-	++	-	+	-	+++
2 mo	-	+	-	+	-	+++
3 mo	-	+	-	+	-	+++

d.. day(s), w.. week(s), m.. month(s)

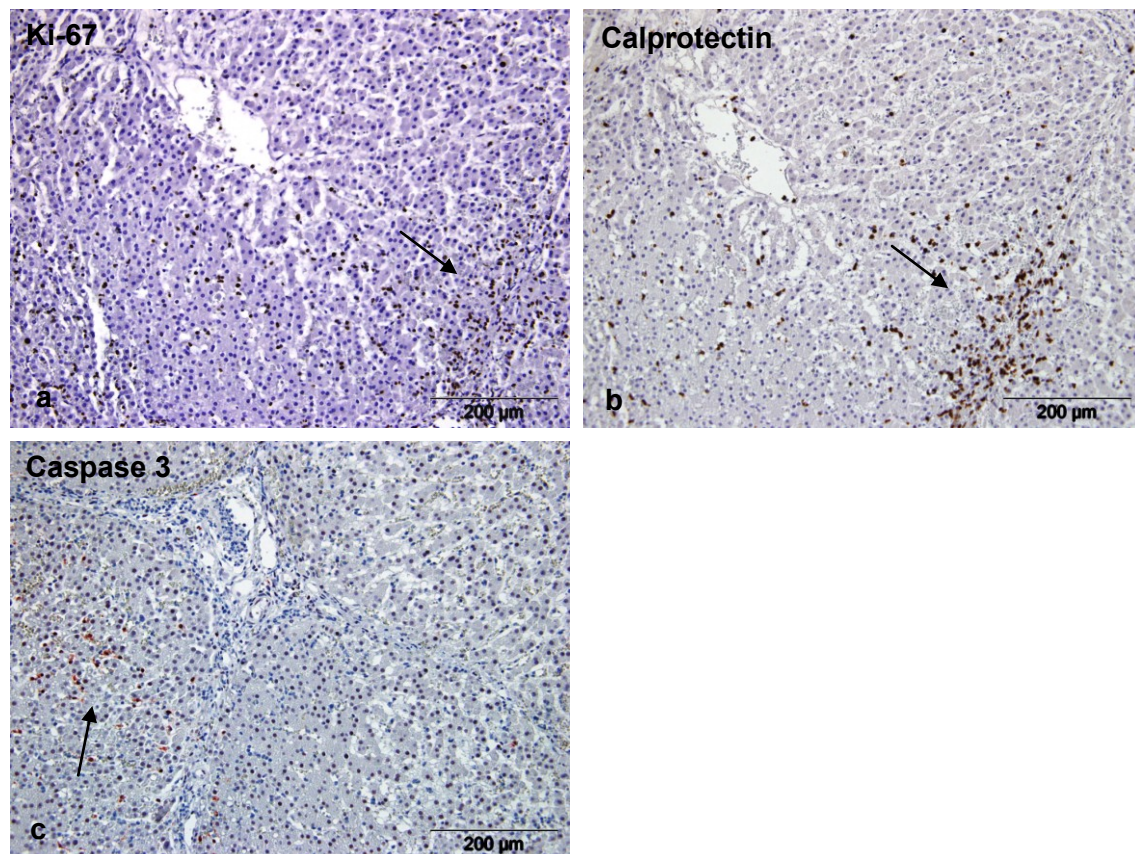


Figure 41: Stained liver tissue of a pig that was euthanized immediately after RFA (0d).

Immediately after RFA we detect proliferating (Ki-67) monocytes/makrophages (Calprotectin) as well as dying hepatocytes (Caspase 3) in the transition zone.

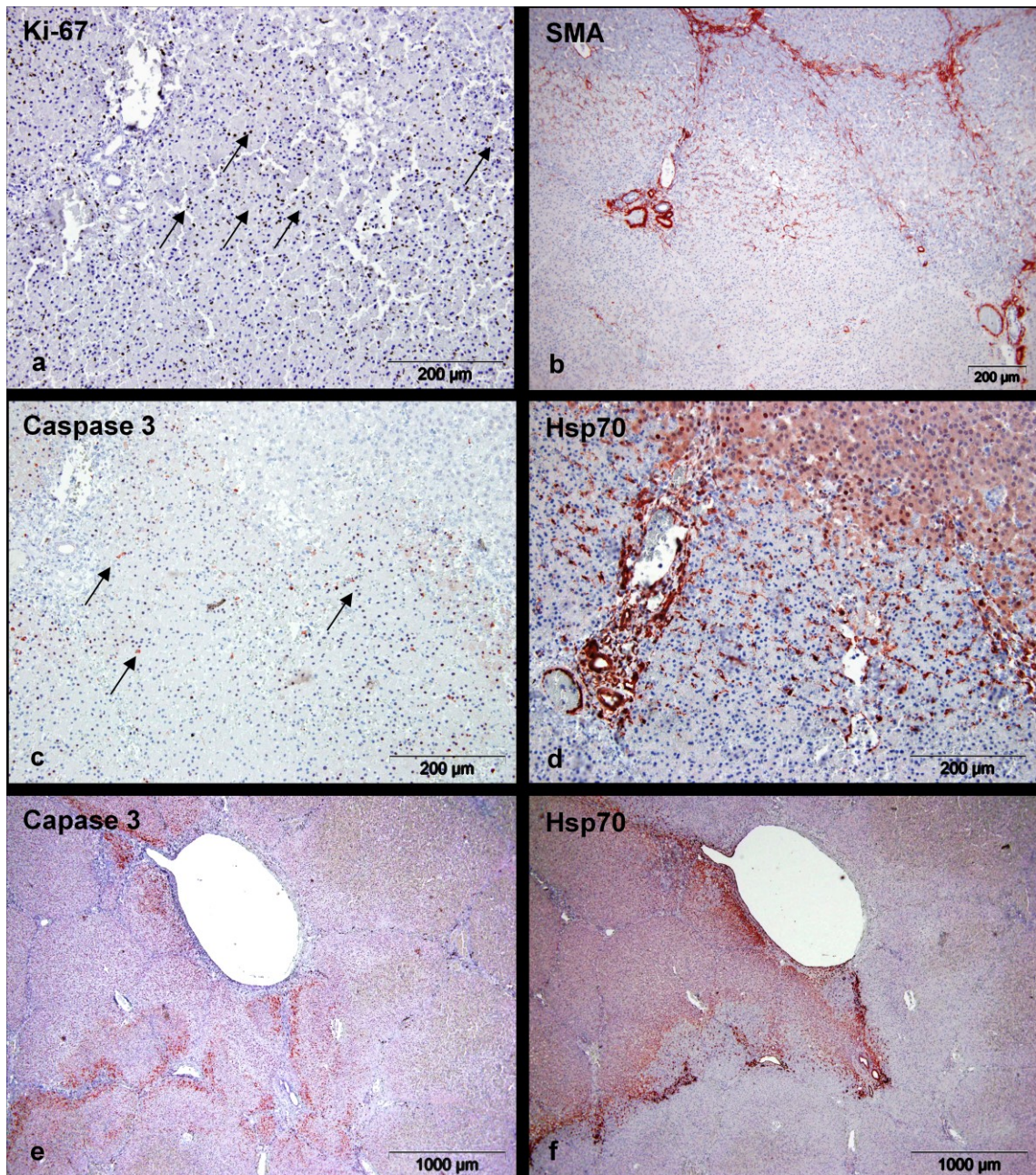


Figure 42: Porcine liver tissue stained one day after RFA (1d).

One day after RFA we found still proliferating (a) and dying cells (c,e). Beside heat stressed hepatocytes migrating fibroblasts (α -SMA staining) express Hsp70 (b, d,f) in the transition zone. Hsp70 high expressing cells co-expressed also Caspase 3.

The transition zone was full of proliferating monocytes even though the numbers decreased as well as the number of apoptotic cells (Figure 43a-c). We could detect only a few activated hepatic stellate cells expressing GFAP (Figure 43d), but many α -SMA positive myofibroblasts, which still co-expressed Hsp70 (Figure 43e-f).

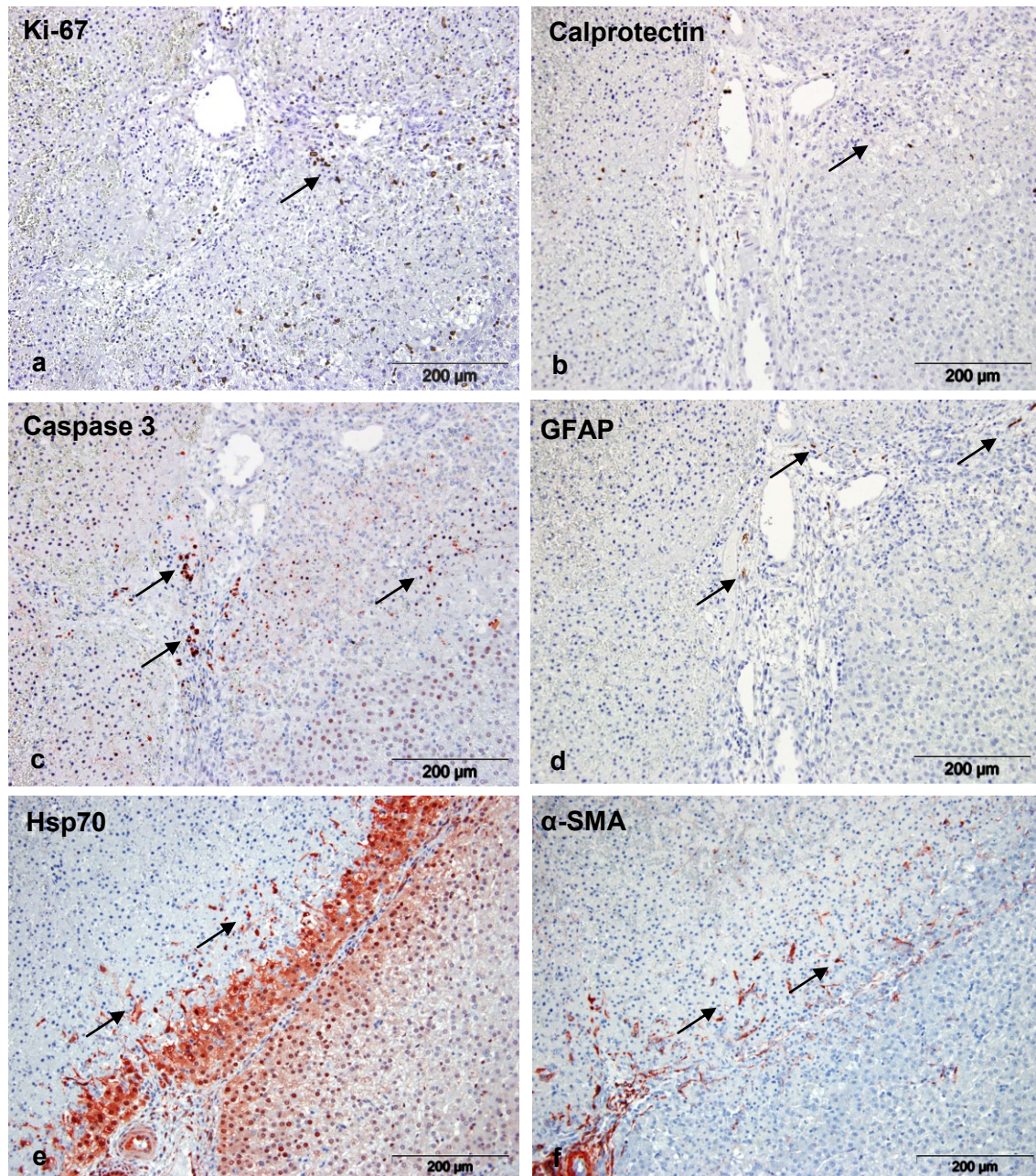


Figure 43: Porcine liver tissue stained two days after RFA (2d).

Two days after RFA we found still proliferating monocytes/macrophages (a,b) and dying hepatocytes (c). Beside heat stressed hepatocytes also migrating fibroblasts (α -SMA staining) express Hsp70 (e,f) in the transition zone. We could detect only a few activated hepatic stellate cells (d).

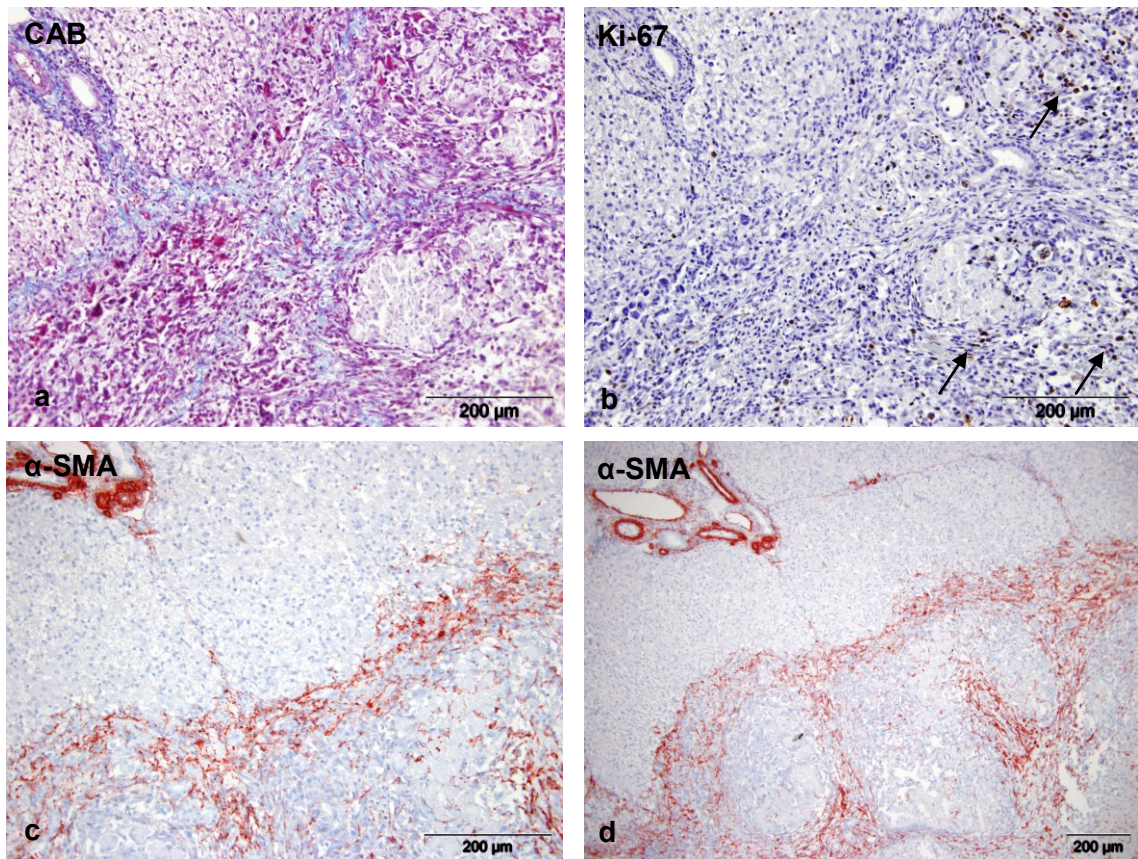


Figure 44: Porcine liver tissue stained one week after RFA (1w).

After one week no apoptotic, but proliferating cells were detected (b). The myofibroblasts form already an α -SMA fibrotic capsule around the lesion (d, f).

After one week (1w) no apoptotic, but proliferating cells were detected (Figure 44b). Myofibroblasts form already an α -SMA fibrotic capsule around the lesion (Figure 44d, f). Two weeks (2w) after ablation the border was characterized by layers of α -SMA positive myofibroblasts. Proliferating monocytes/ macrophages were found mainly within the capsule but also outside. One month (1m) after RFA we still found monocytes and macrophages as well as proliferating hepatocytes (Figure 45a, b). Activated hepatic stellate cells were detected inside the fibrotic capsule (Figure 45c). Furthermore we found Hsp70 positive vessels and histiocytes inside the capsule (Figure 45e, f). Two and three month (2-3m) after RFA we detected Hsp70 positive histiocytes (Figure 46a) and a big fibrotic capsule (Figure 46b-d).

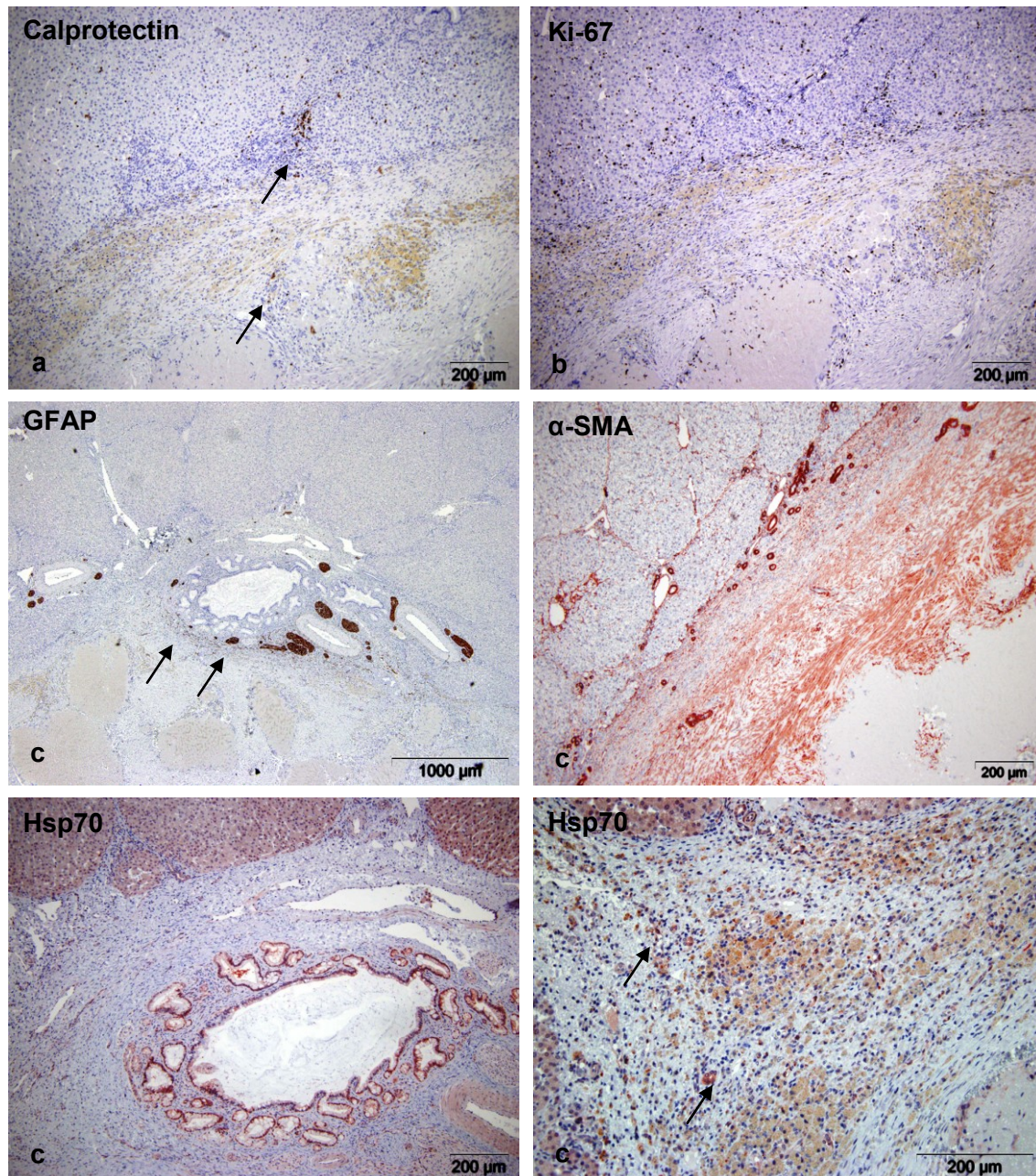


Figure 45: Porcine liver tissue stained one month after RFA (1m).

One month after RFA we still found monocytes and macrophages as well as proliferating hepatocytes (a, b). Activated hepatic stellate cells were detected inside the fibrotic capsule (c). The capsule is surrounded by α-SMA positive myofibroblasts. Furthermore we found Hsp70 positive vessels and histiocytes inside the capsule (e, f).

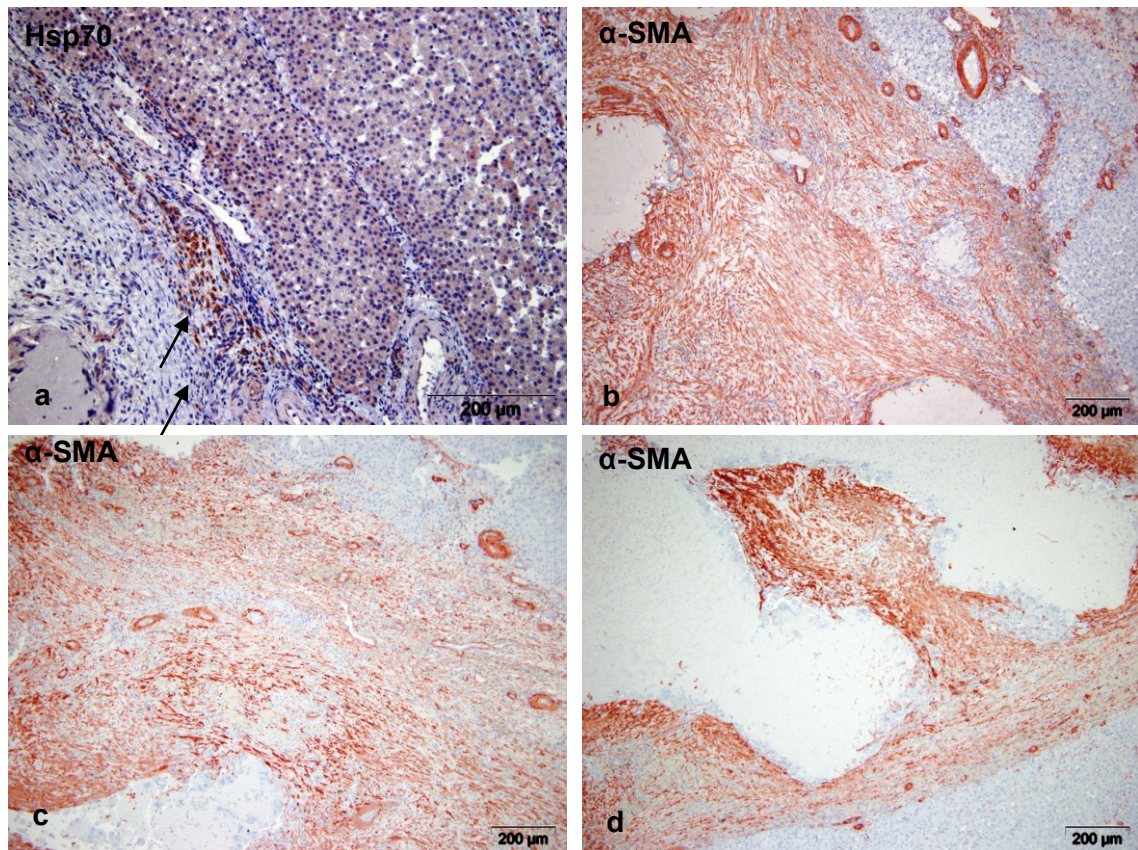


Figure 46: Porcine liver tissue stained two and three months (2m, 3m) after RFA.

Two (a, b) and three (c, d) month after RFA we detected Hsp70 positive histiocytes (a) and a big fibrotic capsule (b-d).

3.7.3 Influence of vessels on RFA outcome

The hepatic flow limits the ablation zone size and can alter the shape of the ablation zone due to the “heat sink” effect. Vessels, which are smaller than 3 mm and ran directly through an ablation, were routinely thrombosed [79].

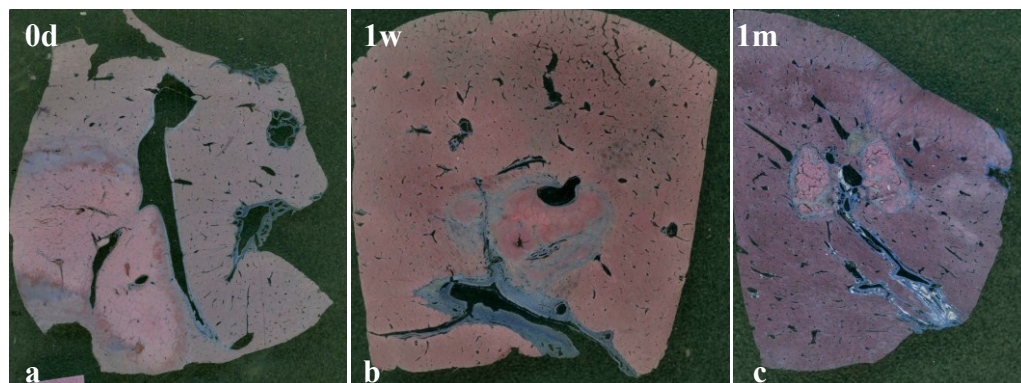


Figure 47: CAB staining of large scale sections.

Heat sink effect caused by big vessels changed the shape of the RFA lesion (a-c).

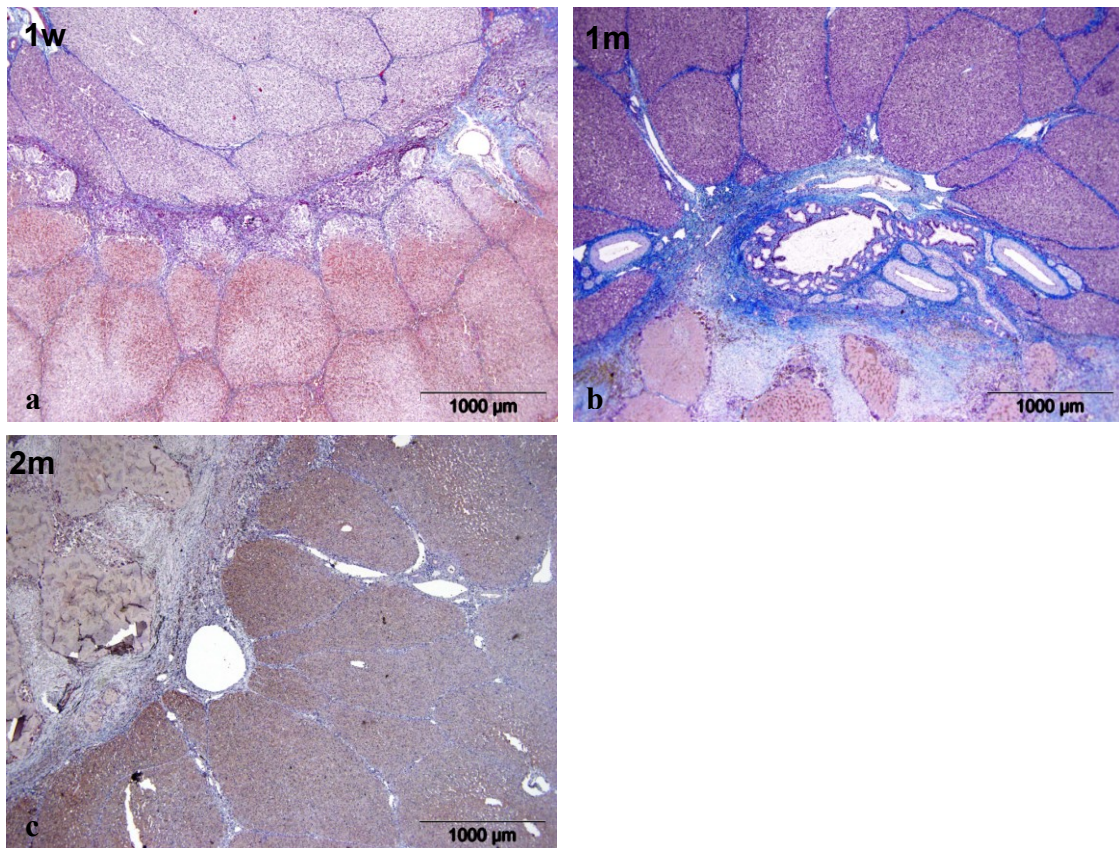


Figure 48: Intact vessels in ablation zone.

Vessels with a diameter greater than 3 mm resist RFA treatment. Pictures show intact vessels in the connective tissue border (a: CAB staining; c: Hsp70) and inside the fibrotic capsule (c: CAB staining)

4 Discussion

HCC accounts for 85% to 90% of primary liver cancers and is the fifth most common malignant tumour in humans [1]. The overall 5-year survival rate of HCC patients in Europe and the United States remain lower than 10% [68]. This tumour normally develops as a consequence of underlying liver disease and often occurs in cirrhotic livers [2].

In contrast to resection and transplantation, RFA is a minimally invasive treatment and can be performed percutaneously in HCC patients. It is the therapy of choice for patients with large, unresectable tumours (due to poor liver function, multifocal disease) or tumours in complicated anatomical locations [79]. RFA uses electromagnetic energy sources to generate heat, ranging from 55°C to 100°C [66]. This heating is strongly influenced by perfusion of the liver (blood flow) and the condition of the organ (healthy, fibrosis, cirrhosis or HCC). It is indispensable to reach lethal temperatures during RFA, because it must be ensured that all tumour cells are destroyed to cure patients and preclude tumour recurrence.

In this study we wanted to investigate the effect of RFA on HCC and healthy liver tissue with the main focus on cell culture experiments simulating different grades of fibrosis and cirrhosis. Moreover, the effect of RFA was evaluated in a large animal model in order to monitor changes in and around the affected area. These results are the basis for creating a bio-heat model, which is essential for computer modelling the RFA process in the liver. Based on this output a patient specific software tool for the RFA should be created which will allow accurate planning and monitoring of the process in clinical applications.

In the course of the cell culture experiments, we wanted to establish a fibrosis model to investigate heat sensitivity and cell survival on different cell lines in order to draw conclusions for an *in vivo* model. We treated MRC-5 cells (lung fibroblast cell line) or LX-1 cells (hepatic stellate cell line) with HepG2 cells (HCC cell line) in co-culture and transwells from 55°C to 100°C for different time spans. After heat exposure, metabolic activity was measured immediately, after 24 h and 48 h using MTS test to assess how many cells survived heating. Furthermore, we investigated the type cell

death after heat exposure using Flow Cytometry in order to distinguish between apoptosis and necrosis.

In the large animal model we performed RFA in pigs and euthanized them after different time spans (0d, 1d, 2d, 1w, 2w, 1m, 2m, 3m). At each time point two animals were treated; one was used for large scale blocks (perfusion fixation), the other one for standard paraffin blocks (immersions fixation). Large scale blocks were sliced and stained for CAB and MB. These blocks gave an overview of the ablation zone and showed the influence of heat sink caused by vessels or gaps between the liver lobes. Additionally, they were used by our EU-partners for 3D reconstruction of serial slices and fusion of the radiology data [118]. Standard paraffin blocks were stained *via* immunohistochemistry for Caspase 3, Hsp70, Ki-67, Calprotectin, GFAP and α -SMA to investigate the changes in liver tissue after RFA.

4.1 Cell culture experiments

We know that temperature tolerance of cells depends on the composition of liver tissue (*e.g.* the grade of fibrosis), among other factors [98], but the exact conditions necessary to induce complete cell death of cancer cells have not yet been studied extensively. Most hyperthermic studies are dealing with temperatures from 42°C-46°C to investigate heat shock and thermotolerance [122], but there is lack of knowledge about heat sensitivity of cells at temperatures ranging from 55°C to 100°C, which are caused during RFA [66]. It was already shown, investigating heat sensitivity of three different HCC cell lines, that too low (not killing) temperatures can induce further malignant transformation of HCCs [90]. Therefore it is essential to create a *in vitro* fibrosis model to gain more information about heat tolerance and cell survival after treatment with high temperatures.

To create our model, we started by heating cell lines MRC-5 and HepG2. MRC-5 is a human lung fibroblast cell line that exhibits the biochemical features of myofibroblasts, which are involved in extracellular matrix accumulation [123, 124]. We chose the HepG2 cell line because it is a common HCC cell line, which contains no hepatitis virus and is not resistant to apoptosis [125]. This cell line was often used for *in vitro* hyperthermia studies [90, 125, 126]. Basile *et al.* showed that hyperthermia at 42°C inhibits cell proliferation and induces apoptosis in HepG2 and Huh7 cells [126]. The

group of Sharma *et al.* investigated the expression of p53 (a tumour suppressor) and Hsp70 in the HCC cell lines HepG2 and Hep3B after heating at 42°C. They found that Hep3B cells are more heat sensitive to heat-induced stress compared to HepG2 cells. In HepG2 cells, heat-induced Hsp70 is regulated by p53 and promotes cells survival in contrast to Hep3B, which are described as p53 defective [125]. As mentioned above, Obara *et al.* investigated the cells lines HepG2, Alexander and Huh7 at different temperatures (from 45°C to 55°C). Then they established sublines from treated cells and investigated them for proliferation rates, heat sensitivity and invasive capacity. They found that even a single heat treatment could induce further transformation of HepG2 cells [90].

Our results show highly significant differences between the temperature tolerance of HepG2 and MRC-5 cells. We observed that the higher the percentage of fibroblasts in the co-culture the more heat was needed to induce cell death. These results clearly demonstrated that HepG2 cells are much more heat sensitive than MRC-5 cells (Figure 16). Furthermore, we found distinct temperature points of cell death (100°C for 5, 10, 15 min, 85°C for 10, 15 min and 75°C for 15 min). 85°C and 100°C led to immediate cell death, because cells die due to protein denaturation, inactivation of protein synthesis, cell cycle progression and DNA repair processes [87, 105]. However, 75°C seemed to be a critical temperature that led not to complete cell death after 10 min. This issue is very important, because even if the target temperature (100°C) is reached near the probe, the temperature decreases within the distance to the needle and is therefore lower in the central or transition zone.

Differences in hepatic and tumour perfusion due to cirrhosis or vessels (heat sink effect) cause variations in size and shape of the lesions [19, 31, 87, 99]. Therefore, not all tumour cells were destroyed and cause tumour recurrence [84-86, 90]. Ruzzenente *et al.* reported a rapid intrahepatic spread of HCC after RFA without metastasis in about 4% of HCC patients after treatment [127]. Furthermore, Obara *et al.* showed in a cell culture study investigating the heat sensitivity of three different HCC cell lines that insufficient RFA, which does not kill all tumour cells, may induce further malignant transformation of HCCs [90]. Portolani *et al.* reported aggressive tumour recurrence after RFA of HCC and metastasis [128]. The group of Seki *et al.* found rapid proliferation and development of hepatic tumours after TACE and RFA in a patient with small HCC. In accordance with MRI, after ablation a complete tumour necrosis was observed and they did not reveal the presence of a tumour around the treated area.

Even the AFP level in serum decreased at the completion of the therapy. But after a short time numerous tumours around the treated area were observed on CT 50 days after RFA. They assumed that the tumour was disseminated through the portal system due to the tumour location [129]. Additionally, Nikfarjam *et al.* and Breitenbuch *et al.* found an altered growth pattern and proliferation in liver metastases after thermal ablation [88, 89].

In our experimental setting, 100% MRC-5 were the thermally most stable cell composition, which led to the conclusion that the higher the severity of liver fibrosis is, the more heat is necessary to cause necrosis. Less sensitivity of fibroblasts compared to hepatocytes could be attributed, among others, to their function as connective tissue. Fibroblasts secrete the precursors of all components of ECM (like e.g. collagens, reticular and elastic fibres), which increases heat tolerance [130, 131]. The reason therefore is so far not described, but it is known that connective tissue influences the outcome of RFA. Livraghi *et al.* reported that fibrotic/cirrhotic liver tissue differs in thermal conductivity from surrounding tissue and acts as thermal isolator with increased heat retention (“oven effect”) [100].

The next step was to create a more physiological model of liver fibrosis [132]. Therefore we chose the human hepatic stellate cell line LX-1. These cells can be activated and stimulated for collagen synthesis by incubating them with 2.5ng/ml TGF- β 1 for 18 h to simulate the surroundings in a cirrhotic liver as described in literature [116]. Additionally, we found that the activation of the TGF- β 1 treated hepatic LX-1 cells led to an increased heat resistance. This could be explained by differentiation of LX-1 into myofibroblasts. They secrete extracellular matrix components due to activation, which confers more heat resistant.

We compared those experiments to our first experiments with the human lung fibroblast cell line MRC-5 and found a very similar survival pattern (Figure 26). We detected that temperature exposure at 85°C for 5 min, 75°C for 10 min and 65°C for 15 min is necessary to induce cell death in all HepG2 and LX-1 cells. In some points the metabolic activity of co-cultures containing MRC-5 cells differ significantly to the co-cultures containing LX-1 cells.

The increased heat resistance possibly could be explained by a different differentiation state of the cells. MRC-5 cells are fully differentiated myofibroblasts with active collagen disposition. In contrast, LX-1 cells are activated hepatic stellate cells, which

are just differentiating into a myofibroblast-like state due to TGF- β 1 as described in literature [6, 115, 116]. It is known that TGF- β 1 activates collagen type I promoters and increases the secretion of ECM proteins such as collagen and fibronectin [133].

Our results showed that HepG2 cells were very sensitive to heat-induced cell death and the sensitivity decreased with rising percentages of MRC-5/ LX-1 cells in the co-culture wells (Figure 16 and Figure 21). Comparing the first to the second experiments we found a higher number of significant differences comparing MRC-5 to HepG2 cells than LX-1 to HepG2 cells, because the metabolic activity of MRC-5 cells was generally higher than that of LX-1 cells. Nevertheless we could show a trend and this supports the findings on a cellular level that different grades of fibrosis affect the outcome of thermal therapy methods. We could not observe significant differences in HepG2 cell survival alone when comparing the first and the second experiment.

The last step was to verify our co-culture model and to create a different model by cultivating the cells separately. The transwell model had the advantage that the metabolic activity could also be measured individually to gain more information about the heat sensitivity of each cell line.

For investigation of cross talk and simulation of *in vitro* fibrosis transwell as well as co-culture models are described in literature. Transwell models are often used to prevent cell interactions and to study soluble factors influencing cell behaviour [134].

For *in vitro* fibrosis studies fresh isolated hepatocytes or HSC as well as rat or human cell lines were used for different scientific questions [135-141]. Some groups even developed three dimensional co-culture models of hepatocytes and HSCs [142-144]. One interesting study was performed by Arnaud *et al.* They found by comparing single culture and co-cultures of hepatocytes and a liver stellate cell line, that the presence of hepatocytes influences the cell proliferation, functionality and ECM production in a negative way [138]. The group of Thomas *et al.* pictured the 3D formation of rat hepatocyte and hepatic stellate cells on poly(DL-lactic acid) surfaces [144]. Using transwell model Sancho-Bru *et al.* investigated the growth, migration and expression behaviour of the HCC cell lines HuH7 (or HepG2) and fresh isolated human HSC. They found a cross talk between HCC cell lines and HSC cells and showed that activated HSC may be stimulated by cancer cells to accumulate and express angiogenic genes [136]. The group of Senoo *et al.* cultivated rat HSCs together with HepG2 cells

in transwells and investigated the release of retinol-binding protein from hepatocytes and its binding and internalization by HSC [137].

We found different levels of cell survival comparing the results of the co-culture [117] and the transwell experiments. Generally, after heating cells at 55°C for 5 and 10 min cultivated in co-cultures than in transwells (Figure 33) showed at short time exposure only marginal differences. Heating cells at 55°C for 15 min seems to be critical for their cell survival and minimal diversion in heat exposure leads deviant results. These conditions show a better survival in transwells than in co-cultures after 48 h. Maybe the cooling due to the additional membrane in transwells biased the measured cell viability. Additionally, we proved that LX-1 cells are more heat tolerant than HepG2 cells. It seems that after heating at 65°C the more LX-1 cells are growing together with HepG2 cells in transwells the lower is the metabolic activity of HepG2 cells (Figure 30 and Figure 31). We traced this phenomenon back to the fact that HepG2 cells are more sensitive when they were seeded in a lower densities (25% = only 2.5×10^4 cells) and thus the heat causes more damage. It seems the higher the cell density the higher the whole metabolic activity after heating HepG2 cells.

However, it was a general finding that cells cultivated together in one well seem to be more heat resistant compared to transwells. We consider that low density and missing cell-cell contact is the main problem in the transwell model. In the liver different cell types are in a united cell structure and are incessantly in contact. Lack of these interactions could affect cells, which could also result apoptosis [145, 146]. So simulation of fibrosis in the co-culture model matches much better with physiological liver tissue than the transwell model. Therefore, we are convinced that the co-culture model is the correct choice for analysing hyperthermia in liver cell lines.

We gained knowledge about the metabolic activity of the cells after heating and drew a conclusion for cell survival, but we also needed to know if the cells die due to apoptosis or necrosis. The necrotic way of cell death leads to inflammation due to uncontrolled release of intracellular content [121]. This inflammation could negatively influence the wound healing after RFA and the survival of the patients. It is already known that application and central zone after RFA are caused by necrotic coagulation effected by high temperatures [91]. We wanted to investigate how the cells react in the transition zone, where non lethal temperatures occur during ablation.

Preliminary, we also tested Flow Cytometry measurement of Caspase 3. We used a few methods to induce apoptosis (heating, camptothecin and staurosporine), but we were not able to get meaningful results. Then we choose Annexin V staining, which it is already used to distinguish between apoptosis and necrosis. It is known that in contrast to necrosis during apoptosis a time lag exists between externalization of PS and membrane permeabilization (the viability dye uptake) [121, 147].

It was very complex to optimise the protocol for characterisation of cell death after heat exposure. We wanted the cells to grow together in one single well, because based on our prior experience we know, that heat sensitivity of HepG2 cells is influence by the seeding density. For this reason we used an intercellular staining method to distinguish LX-1 and HepG2 cells in co-culture. The Vybrant® DiO and DiD cell-labelling dyes suited best for Flow Cytometry measurement and could also be used for imaging (Figure 35). Because DiO (green, similar to FITC) and DiD (red, similar to APC) we had to chose Annexin V and the viability dye in the colours yellow (Annexin V PE) and blue (eFlour 450, similar to pacific blue) to avoid overlapping of the emission spectra.

All investigated time points (55°C 15 min, 65°C 10 min, 65°C 15 min and 75°C 5 min) showed apoptotic cell death (Figure 34). We detected no differences in cell survival or death of the different co-cultures and therefore we calculated one single value per cell line. Analysing the data we found a continuous level of early apoptotic (Annexin V positive, Figure 34) LX-1 and HepG2 cells. These cells were damaged during normal detaching procedure as it also occurs during cultivation. After heating at 65°C for 15 min we detected a striking peak and a high standard error after 8 h for which we have no explanation. Possibly, this peak is caused by minimal thermal deviation during the heating.

Comparing MTS (Figure 21) to Flow Cytometry data (Figure 38) we found distinctions in cell viability after heating. Using Flow Cytometry the differences found in cell survival after heating at 55°C for 15min were similar, even if the viability was about 20% higher. Heating at 65°C for 10 min showed no observable differences in cell survival comparing the two methods. However, heating at 65°C for 15 min displayed a clearly higher viability measured *via* Flow Cytometry (60%-90%) compared to MTS test (10%-60%). With both methods a decrease of the viability after 8 h-24 h was detected. Heating at 75°C for 5 min showed similar cell survival after heating and stayed constant after 24h according to MTS test. However, Flow Cytometry detected a decrease of viability after 24h. These differences can't be caused by deviation in

heating, because immediately after heating the data are very similar. Possibly an methodical error occurred and ingredients of the cell culture medium were destroyed or became toxic during heating (Figure 38). HepG2 cells seem to be more sensible for this ingredients then LX-1 cells.

In summary the results comparing Flow Cytometry and MTS were very similar, but some differences occurred. These could possibly be explained by deviations in heating or cell behaviour. For a more detailed comparison of the two methods more time points and replications would be needed. However, to clarify the way of cell death, which was the main aspect of the experiments, the Flow Cytometry experiments were sufficient. These data also correlate to below-mentioned histological results. By means of Caspase 3 and Calprotectin we found inflammation caused by apoptotic cells in the transition zone.

4.2 Porcine liver tissue after RFA

Pigs are often used for experimental hepatic surgery and treatment experiments in liver diseases [25, 148]. We chose the pig model for this experimental setting, because porcine liver strongly resemble human conditions. However, it appears as if the liver lobes but also individual liver segments are much more moveable in pigs than in humans. At such margins, heat transfer can be substantially lower. These transition regions are not always clearly visible on CT images, which make RFA treatment more complicated and leads to ablation zones with atypical shapes. In the human liver, this effect is less pronounced. Furthermore, it has to be mentioned that younger animals (2-5 months old pigs) have a better liver regeneration, which causes a rapid shrinking of the ablated region. In our experiments we used healthy pigs and therefore these results can't be compared directly to patients of older age that suffer from HCC or liver metastases. As a result of the limited capacity for regeneration in these humans, the size of the ablated region decreases slowly after RFA [101, 149, 150]. Morimoto *et al.* showed a decrease of the ablation zone compared to the pre-treated HCC tumours after 24 weeks follow up CT scan [150]. Kuehn *et al.* found significant decreases in the size of the necrosis zone after LITT in patients with liver metastases after three month [101]. In contrast the group of Feliberti *et al.* reported a nearly equal size of the ablation zone after RFA in liver metastases [149]. Maybe this controversy is caused by not reported variation in tumour size or age of the patients. Possibly, even the surrounding

tissue (healthy or cirrhotic) could have an influence on the further development of the lesion. However, we aimed to characterize the encapsulation of the necrotic area and could gain useful insights using the pig model. The different extent for capsule development comparing humans to pigs does not affect our results.

In this study we investigated the development of the fibrosis capsule after RFA over a time period of three months. Thereby the main focus was on apoptosis, inflammation and encapsulation (wound healing) of the transition zone.

To support our Flow Cytometry data (chapter 3.6) we also investigated the treated liver tissue for apoptosis using a Caspase 3 antibody. Generally, we could demonstrate that the cells damaged after RFA need up to two days to undergo apoptosis (0d up to 2d). These data relate to the study of Nikfarjam *et al.* They induced local hypothermia via laser in CBA strain mice with liver metastases and sacrificed them after different time spans (0 h, 12 h, 24 h, 48 h, 72 h, 120 h and 168 h after treatment). Their study showed apoptosis in liver tissue the following 72 h, peaking after 12 h, through activated Caspase 3 staining. They also mentioned that TUNEL labelling, another method for detection of apoptosis, was less specific than staining for activated Caspase 3 [151]. All together, these results correlate with our *in vitro* data (Flow Cytometry) and show that in the transition zone of the lesion cells are apoptotic due to non lethal temperatures.

By staining for heat shock protein Hsp70, we wanted to picture the heat stress in surrounding liver tissue after RFA. It is known that Hsps are involved in denaturation, folding, activation and transport of other proteins and synthesized under stressful conditions [152]. These proteins play a major role in tumour immunogenicity. They activate resting antigen-presenting cells to take up and process the tumour antigens and up-regulate the expression of co-stimulatory molecules necessary for T-cell activation [105]. In contrast to Rai *et al* we found a strong expression of Hsp70 in the transition zone (1d and 2d), but also weak staining in apparent healthy liver tissue (Figure 43f) [105]. They found maximum expression in the transition zone and no Hsp70 expression in the healthy surrounding liver tissue five days after RFA in white land-race pigs. In our study, this pattern could not be unspecific staining since immediately after RFA (0d) no Hsp70 positive cells were detectable. It seems that these cells are injured after RFA, but the proceeded hyperthermia was not high enough to kill these cells but only to stress them. Rai *et al* suggested that Hsp70 expression can also be stimulated by hyperthermic sub lethal conditions. Additionally, it is also known that Hsp70 is able to

inhibit apoptosis thereby increasing the survival of cells due to their chaperone activity [153]. This could protect cells from thermal stress by neutralizing the toxic effects of denaturated proteins [154].

On this basis we conclude that heat shock protein expression in healthy tissue surrounding the lesion is an evidence for hyperthermic stress. Interestingly it is not limited to the ablated area and could also be found in the surrounding healthy tissue. Therefore, we estimate that the strong Hsp70 positive cells in the transition zone undergo apoptosis in contrast to only heat stressed not apoptotic cells in the surrounding healthy tissue. Furthermore, it is known that Hsp70 is most strongly expressed (under all Hsps) after heat exposure and it is speculated that they are initiating innate immunity *in vivo* [152]. This effect seems to be reinforced inflammation caused by apoptosis and explain also the co-expression of Hsp70 and Caspase 3 in cells of the transition zone (Figure 42e, f).

To investigate the inflammation process after RFA treatment we were looking for a marker characterizing macrophages in the transition zone. We tested a few antibodies for porcine CD68, which is a classical marker for macrophages, but none of them was suitable. Thereafter we chose the monocyte, macrophage and neutrophils marker Calprotectin. This protein has antibacterial, antifungal, immunomodulating and antiproliferative effects and is a potent chemotactic factor for neutrophils. It can be detected in neutrophils, monocytes, macrophages and squamous mucosal epithelia and has been shown as an important marker for identifying macrophages in tissue sections (www.abcam.com/ab75913.html; abcam, UK). Calprotectin positive cells were present in different levels over the whole three months (0d up to 3 m). Their presence immediately after treatment assumes that these are tissue specific macrophages (Kupffer cells) that become activated immediately. Their role in the liver could be described by phagocytosis, processing of ingested material and antigens, secretion of biological products, tumour surveillance and liver regeneration [155]. This explains probably why we could still detect macrophages after three months. Based on our immunohistochemistry, we could not find an increase of macrophages or monocytes after treatment. According to literature, the amount of macrophages increases for a short time after 48 h [110], but we could not observe this due to non investigated time points after RFA. However, we could detect histiocytes one month after RFA within the fibrotic boundary and not inside the necrotic area. These cells are formed by macrophage fusion (also called giant cells) and described as foreign body reaction [156,

157]. Foreign body reaction was often described after renal RFA [158-160]: Only a few groups reported foreign body reaction in liver after RFA. Kasper *et al.* speculated that the transition zone acts as an inflammatory border. He thought that heat could have changed the protein configuration and created a foreign body structure [156]. This thermally fixed tissue appears to resist tissue repair and resolution. This lack of tissue degradation may result from a resistance to capillary and inflammatory cell penetration into the ablation zone [161]. Nevertheless, the lesion shrinks over time when the necrotic material is absorbed by the body [101, 150, 150]. This reaction lasts longer in patients than in young pigs.

Generally, we could say that RFA causes acute inflammatory reaction and the treated tissue is encapsulated very fast. If the tumour cells are killed completely, the ablated area will vanish over years and will cause no further complications in patients.

We investigated the development of the fibrosis capsule after RFA over a time period of three months. At the beginning three different zones were clearly specifiable. In the centre of the lesion we found coagulation necrosis with loss of nuclear staining (application zone, Figure 40). Additionally the healthy liver tissue (reference zone) was separated clearly from the central zone after treatment by a border of hemorrhagic rim (transition zone; Figure 39a). After one week the lesion developed a fibrotic capsule (blue rim), still followed by a red rim containing erythrocytes (Figure 40d, Figure 39d). After two weeks the bleeding disappeared and the fibrotic rim became larger (Figure 40e, Figure 39e). In meantime the RFA lesion began to shrink until it was hard to detect. Interestingly, the granulation tissue did not invade the necrotic area like the inflammatory cells. We wanted to clarify if the encapsulation is triggered by HSC (comparable with fibrogenesis) or by migrating fibroblasts (comparable with dermal wounds).

GFAP was described as an early marker for hepatic stellate cell activation [119, 162-164]. To differ between HSC and other fibroblasts we stained the liver tissue for GFAP (HSC) and α -SMA (all myofibroblasts). However, we could not detect (an increasing amount of) GFAP positive HSC, but an increasing population of α -SMA positive myofibroblasts from the first day till they formed a big fibrosis capsule (1d up to 3m). In rat liver studies it was found that GFAP is expressed in quiescent HSC and declines with activation or is expressed early in HSC activation but declines with increasing α -SMA expression [165]. So the precise role and temporal significance of GFAP in

hepatic stellate activation in human liver is at the moment not clearly understood. Furthermore Knittel *et al* identified an α -SMA negative myofibroblast subpopulation in liver tissue, so it would be better to characterise the whole myofibroblast cell population with vimentin than with α -SMA [164]. Kobold *et al* described another possible marker for hepatic stellate cells called reelin, which is an extracellular matrix protein that is only present in HSC [166]. Vinculin, a cytoskeletal protein involved in membrane-cytoskeleton interactions, is also specific for HSC [167]. But with both markers it is not possible to distinguish between quiescent and activated HSC.

We could not identify the origin of the fibrous capsule, but we know that it is caused by collagen producing fibroblasts. However, it seems to be caused by replacement of the transition zone [150].

4.3 Conclusion

In this study we wanted to gain more information about the physiological parameters influencing RFA and picture the outcome over three months by means of cell culture and animal experiments.

We demonstrated that fibrosis influences the shape and the size of a RFA lesion at least that much as blood flow (“heat sink” effect). Therefore, strategies to estimate the grade of fibrosis/cirrhosis before RFA as accurately as possible should be an intention. This could keep patients from incalculable complications. Moreover, to prevent further tumour growth or alteration it is absolutely necessary to create a boundary rim in healthy surrounding tissue. Consequently, a temperature of at least 75°C must be reached to destroy all tumour cells.

The cells inside the ablation area are thermally fixed or die by necrosis, which leads to an acute inflammatory reaction. This dead tissue appears to resist wound repair and resolution. In contrast, cells in the transition zone die apoptotic and become replaced by fibroblasts. They produce a fibrotic capsule, which acts as inflammatory border and causes foreign body reaction. In contrast to wound healing, foreign body reaction is associated with a chronic local inflammation, which could be responsible for absorption of the necrotic material and for shrinkage of the lesion in the course of time. However, we aimed to characterize the encapsulation of the necrotic area and could gain useful insights using the pig model.

Generally, RFA causes acute inflammatory reaction due to wounding of liver tissue. A capsule is formed around the ablation zone, which reacts as inflammatory border and causes foreign body reaction. If the tumour cells are killed completely within the RFA, the ablated area will vanish over years and will cause no further complications.

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6 Appendix

6.1 Figures

Figure 1: Hepatocyte apoptosis induces hepatic fibrosis.	4
Figure 2: Characteristics of activated HSC.	5
Figure 3: Different zones of a RFA lesion	15
Figure 4: Presence of the different cells during wound healing process.	16
Figure 5: Co-Culture of LX-1 and MRC-5 with HepG2.	22
Figure 6: Cultivating HepG2 and LX-1 cells in transwells.	23
Figure 7: Cells prepared for transwell experiment:	23
Figure 8: Cells prepared for experiments:	24
Figure 9: Measurement of cell metabolism via MTS test.	24
Figure 10: Scheme for seeding of HepG2 and LX-1 co-cultures 18 h before heating	25
Figure 11: Pigs (suus scrofa) for RFA treatment standing in their box.	29
Figure 12: Rita probe and generator, scalable spherical ablations.	31
Figure 13: Preparation, ablation and liver explantation of a pig.	32
Figure 14: RFA area after perfusion fixation.	33
Figure 15: Comparison of a large scale and a standard paraffin block and slide.	35
Figure 16: Shift of viabilities after heating experiments ranging from 5 - 15 minutes. ..	42
Figure 17: Fluorescence staining of cell viability.	45
Figure 18: Heating of MRC-5, HepG2 cells and their co-cultures for 5 minutes.	46
Figure 19: Heating of MRC-5, HepG2 cells and their co-cultures for 10 minutes.	48
Figure 20: Heating of MRC-5, HepG2 cells and their co-cultures for 15 minutes.	49
Figure 21: Shift of metabolic activity after hyperthermia ranging from 5 - 15 minutes.	50
Figure 22: Differences in cell death induced via hyperthermia.	53
Figure 23: Heating LX-1, HepG2 cells and co-cultures for 5 minutes.	54
Figure 24: Heating LX-1, HepG2 cells and co-cultures for 10 minutes.	55
Figure 25: Heating LX-1, HepG2 cells and co-cultures after 15 minutes.	56
Figure 26: Shift of metabolic activity after hyperthermia ranging from 5 - 15 minutes.	57
Figure 27: Heating in transwells comparing LX-1 and HepG2	59
Figure 28: Shift of metabolic activity after hyperthermia ranging from 5 - 15 minutes (HepG2).	60

Figure 29: Shift of metabolic activity after hyperthermia ranging from 5 - 15 minutes (LX-1).	61
Figure 30: Heating HepG2 and LX-1 cells for 5 min in transwells.	64
Figure 31: Heating HepG2 and LX-1 cells for 10 min in transwells.	65
Figure 32: Heating HepG2 and LX-1 cells for 15min in transwells.	66
Figure 33: Comparing co-culture and transwell heating experiments.	67
Figure 34: Apoptotic cell death after heating at 65°C for 15 min (Flow Cytometry). ...	68
Figure 35: Intracellular staining of HepG2 and LX-1 cells in co-culture.	69
Figure 36: Percentage of cells in early apoptosis after heating (Flow Cytometry).	70
Figure 37: Percentage of cells in late apoptosis after heating (Flow Cytometry).	71
Figure 38: Percentage of viable cells after heating (Flow Cytometry).	72
Figure 39: CAB staining of survivor pigs (immediately after RFA up to 3 months).	74
Figure 40: CAB staining of Survivor pigs.	75
Figure 41: Stained liver tissue of a pig that was euthanized immediately after RFA (0d).	76
Figure 42: Porcine liver tissue stained one day after RFA (1d).	77
Figure 43: Porcine liver tissue stained two days after RFA (2d).	78
Figure 44: Porcine liver tissue stained one week after RFA (1w).	79
Figure 45: Porcine liver tissue stained one month after RFA (1m).	80
Figure 46: Porcine liver tissue stained two and three months (2m, 3m) after RFA.	81
Figure 47: CAB staining of large scale sections.	81
Figure 48: Intact vessels in ablation zone.	82

6.2 Tables

Table 1: Overview of potential serum marker for HCC [21].	7
Table 2: Cell culture material and reagents	28
Table 3: Pigs survived for different time spans after RFA	30
Table 4: Protocol for RFA for a single lesion.	32
Table 5: Antibodies and other reagents used for immunohistochemistry.	41
Table 6: Percentages of viable HepG2 and MRC-5 cells in heating experiments.	43
Table 7: Percentages of viable HepG2 and LX-1 cells in heating experiments.	52
Table 8: Results of transwell experiments (HepG2).	62
Table 9: Results of transwell experiments (LX-1).	63
Table 10: Antibody staining in transition zone after RFA.	76

6.3 Abbreviations

bFGF	basic fibroblast growth factor
CAB	chromotrope anilinblue
CT	Computer tomography
EGF	epithermal growth factor
FGF	fibroblast growth factor
HCC	hepatocellular carcinoma
HSC	Hepatic stellate cells
LITT	laser-induced thermotherapy
MB	methylene blue
MRI	magnetic resonance imaging
PDGF	platelet derived growth factor
PEIT	percutaneous ethanol injection therapy
PMCT	percutaneous microwave coagulation therapy
RFA	radiofrequency ablation
TACE	transcatheter arterial chemoembolization
TGF- α	transforming growth factor alpha
TGF- β	transforming growth factor beta
TNF- α	tumour necrosis factor alpha
US	Ultrasonography

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6.5 Staining protocols for immunohistochemistry

~ *Activated Caspase 3 (R&D Systems, AF835)*

using UltraVision LP

Antibody was dissolved in 500µl 1x PBS, aliquoted and frozen at 20°C

- Preparation of paraffin-embedded sections for immunohistochemistry in sections of 2µm, mounting sections onto slides and drying overnight
- Next day the slides were heated for 30 min at 70°C in a heating cabinet.
- Rehydration and deparaffination of the tissue sections (xylene, isopropanol)
- Wash 2 times in 1x PBS.
- Antigen demasking using microwave: Slides are boiled in 0.1M Citrate buffer, pH 6.0 at 150 watt for 40 min. Finally the slides are cooled down for 20 min to room temperature.
- Wash 4 times in 1x PBS.
- Blocking of endogenous peroxidase for 15' in 3% H₂O₂-Methanol at room temperature.
- Ultra UV-Blocking for 10 min. Don't wash the slides, immediately incubate with primary antibody
- Incubation with activated Caspase 3 (R&D Systems AF835) in a dilution of 1:25 in Dako Antibody Diluent for 1 hour at RT
- Wash 4 times in 1x PBS
- Incubation with Primary Antibody Enhancer for 10 min
- Wash 4 times in 1x PBS
- Incubation with HRP-Polymer for 30 min in the dark (NOTE: HRP Polymer is light sensitive. Please avoid unnecessary light exposure and store in opaque vial.)
- Wash 4 times in 1x PBS
- Visualization is achieved using the chromogen AEC for 8 min
- Reaction is stopped washing with 1x PBS
- Counterstaining is achieved with Hematoxylin
- Slides are coverslipped with Aquatex.

~ **HSP70 (abcam, ab31010)**

using UltraVision LP

- Preparation of paraffin-embedded sections for immunohistochemistry in sections of 2µm, mounting sections onto slides and drying overnight
- Next day the slides were heated for 30 min at 70°C in a heating cabinet.
- Rehydration and deparaffination of the tissue sections (xylene, isopropanol)
- Wash 2 times in 1x PBS.
- Antigen demasking using microwave: Slides are boiled in 0.1M Citrate buffer, pH 6.0 at 150 watt for 15 min. Finally the slides are cooled down for 20 min to room temperature.
- Wash 4 times in 1x PBS
- Blocking of endogenous peroxidase for 15' in 3% H₂O₂-Methanol at room temperature.
- Ultra UV-Blocking for 10 min. don't wash the slides, immediately incubate with the primary antibody
- Incubation with Hsp70 (abcam ab31010) in a dilution of 1:100 in Dako Antibody Diluent for 1 hour at RT
- Wash 4 times in 1x PBS
- Incubation with Primary Antibody Enhancer for 10 min
- Wash 4 times in 1x PBS
- Incubation with HRP-Polymer for 30 min in the dark
- Visualization is achieved using the chromogen AEC for 8 min
- Reaction is stopped washing with 1x PBS
- Counterstaining is achieved with Hematoxylin
- Slides are coverslipped with Aquatex.

~ **Ki67 (Dako, M7240)**

using UltraVision LP

- Preparation of paraffin-embedded sections for immunohistochemistry in sections of 2µm, mounting sections onto slides and drying overnight
- Next day the slides were heated for 30 min at 70°C in a heating cabinet.
- Rehydration and deparaffination of the tissue sections (xylene, isopropanol)
- Wash 2 times in 1x PBS.
- Antigen demasking using microwave: Slides are boiled in 0.1M Citrate buffer, pH 6.0 at 150 watt for 40 min. Finally the slides are cooled down for 20 min to room temperature.
- Wash 4 times in 1x PBS
- Blocking of endogenous peroxidase for 15' in 3% H₂O₂-Methanol at room temperature.
- Ultra UV-Blocking for 10 min. don't wash the slides, immediately incubate with the primary antibody.
- Incubation with Ki67 Clone MIB-1 (Dako, M7240) in a dilution of 1:200 in Dako Antibody Diluent for 1 hour at RT
- Wash 4 times in 1x PBS
- Incubation with Primary Antibody Enhancer for 10 min
- Wash 4 times in 1x PBS
- Incubation with HRP-Polymer for 30 min in the dark
- Wash 4 times in 1x PBS
- Visualization is achieved using the chromogen DAB for 8 min
- Reaction is stopped washing with 1x PBS
- Counterstaining is achieved with Hematoxylin
- For dehydration, tissues were put in rising concentrations of isopropanol and xylene.
- Slides are coverslipped with GLC Mounting Medium

~ ***GFAP (Thermo Scientific, MS-280-P0)***

using UltraVision LP

- Preparation of paraffin-embedded sections for immunohistochemistry in sections of 2µm, mounting sections onto slides and drying overnight
- Next day the slides were heated for 30 min at 70°C in a heating cabinet.
- Rehydration and deparaffination of the tissue sections (xylene, isopropanol)
- Wash 2 times in 1x PBS.
- Antigen demasking using microwave: Slides are boiled in 0.1M Citrate buffer, pH 6.0 at 150 watt for 30 min. Finally the slides are cooled down for 20 min to room temperature.
- Wash 4 times in 1x PBS
- Blocking of endogenous peroxidase for 15' in 3% H₂O₂-Methanol at room temperature.
- Ultra UV-Blocking for 10 min. don't wash the slides, immediately incubate with the primary antibody.
- Incubation with GFAP (Glial Fibrillary Acidic Protein) Ab-1 (Clone GA-5) in a dilution of 1:500 in Dako Antibody Diluent for 1h at RT
- Wash 4 times in 1x PBS
- Incubation with Primary Antibody Enhancer for 10 min
- Wash 4 times in 1x PBS
- Incubation with HRP-Polymer for 30 min in the dark
- Wash 4 times in 1x PBS
- Visualization is achieved using the chromogen DAB for 5 min
- Reaction is stopped washing with 1x PBS
- Counterstaining is achieved with Hematoxylin
- For dehydration, tissues were put in rising concentrations of isopropanol and xylene.
- Slides are coverslipped with GLC Mounting Medium

~ ***Calprotectin (Thermo Scientific, MS-180-P0)***

using UltraVision LP

- Preparation of paraffin-embedded sections for immunohistochemistry in sections of 2µm, mounting sections onto slides and drying overnight
- Next day the slides were heated for 30 min at 70°C in a heating cabinet.
- Rehydration and deparaffination of the tissue sections (xylene, isopropanol)
- Wash 2 times in 1x PBS.
- Antigen demasking using 1mg/ml Trypsin: The slides were incubated in a 37°C waterbath for 15min
- Wash 4 times in 1x PBS
- Blocking of endogenous peroxidase for 15' in 3% H₂O₂-Methanol at room temperature.
- Ultra UV-Blocking for 10 min. don't wash the slides, immediately incubate with the primary antibody.
- Incubation with Calprotectin-Monocyte/ Macrophage/ L1 Protein Ab-1 (Clone MAC 387) in a dilution in Dako Antibody Diluent 1:500 for 1h at RT
- Wash 4 times in 1x PBS
- Incubation with Primary Antibody Enhancer for 10 min
- Wash 4 times in 1x PBS
- Incubation with HRP-Polymer for 30 min in the dark
- Wash 4 times in 1x PBS
- Visualization is achieved using the chromogen DAB for 5 min
- Reaction is stopped washing with 1x PBS
- Counterstaining is achieved with Hematoxylin
- For dehydration, tissues were put in rising concentrations of isopropanol and xylene.
- Slides are coverslipped with GLC Mounting Medium

~ *α -SMA (Sigma Aldrich, A2547)*

using UltraVision LP

- Preparation of paraffin-embedded sections for immunohistochemistry in sections of 2 μ m, mounting sections onto slides and drying overnight
- Next day the slides were heated for 30 min at 70°C in a heating cabinet.
- Rehydration and deparaffination of the tissue sections (xylene, isopropanol)
- Wash 2 times in 1x PBS.
- No antigen demasking
- Blocking of endogenous peroxidase for 15' in 3% H₂O₂-Methanol at room temperature.
- Ultra UV-Blocking for 10 min. don't wash the slides, immediately incubate with the primary antibody.
- Incubation with α -SMA antibody 1A4 (A2547, Sigma Aldrich) in a dilution 1:5000 in Dako Antibody Diluent for 1h at RT (aliquoted stored at -20°C).
- Wash 4 times in 1x PBS
- Incubation with Primary Antibody Enhancer for 10 min
- Wash 4 times in 1x PBS
- Incubation with HRP-Polymer for 30 min in the dark
- Wash 4 times in 1x PBS
- Visualization is achieved using the chromogen AEC for 8 min
- Reaction is stopped washing with 1x PBS
- Counterstaining is achieved with Hematoxylin
- Slides are coverslipped with Aquatex.

6.6 Publications

Mayrhauser, U; Stiegler, P; Stadlbauer, V; Köstenbauer, S; Leber, B; Konrad, K; Iberer, F; Tscheliessnigg, K; **Cell interactions influence Effect of Hyperthermia on Liver Cell Lines.** Anticancer Research (accepted in Anticancer Research).

Mayrhauser, U; Stiegler, P; Stadlbauer, V; Köstenbauer, S; Leber, B; Konrad, K; Iberer, F; Portugaller, RH; Tscheliessnigg, K **Effect of Hyperthermia on Liver Cell Lines: Important Findings for Thermal Therapy in Hepatocellular Carcinoma.** Anticancer Research, 2011; 31:1583-1588

Koestenbauer, S; Stiegler, P; Stadlbauer, V; Mayrhauser, U; Leber, B; Blattl, D; Kainz, B; Reich, O; Portugaller, RH; Wiederstein-Grasser, I; Tscheliessnigg, KH **Visualization of large-scale sections.** Journal of Surgical Radiology . 2011; 2(2):

O'Neill, DP; Peng, T; Stiegler, P; Mayrhauser, U; Koestenbauer, S; Tscheliessnigg, K; Payne, SJ **A Three-State Mathematical Model of Hyperthermic Cell Death.** Ann Biomed Eng. 2010.

Alhonnoro, T; Pollari, M; Lilja, M; Flanagan, R; Kainz, B; Muehl, J; Mayrhauser, U; Portugaller, H; Stiegler, P; Tscheliessnigg, K **Vessel segmentation for ablation treatment planning and simulation.** Med Image Comput Comput Assist Interv. 2010; 13(Pt 1):45-52.

Effect of Hyperthermia on Liver Cell Lines: Important Findings for Thermal Therapy in Hepatocellular Carcinoma

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Running title: Mayrhauser *et al*: Effect of Hyperthermia on Liver Cell Lines

This is an experimental study

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Abstract. *Background: Hepatocellular carcinoma is the fifth most common malignant tumour, with a high mortality rate. This study aimed to investigate the effect of hyperthermia on HepG2 and LX-1 cell lines to gain more information on thermal treatment of liver tumours. Material and Methods: The cell lines HepG2, LX-1 and their co-cultures were heated from 55°C to 85°C for different time spans. After heat exposure, metabolic activity was measured immediately, and after 24 h and 48 h using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) test to assess how many cells had survived heating. Results: Our results show highly significant differences between the temperature tolerance of HepG2 and LX-1 cells. Alone, HepG2 cells are most sensitive to heat-induced cell death, their sensitivity decreased with rising percentages of LX-1 cells in the co-culture. Conclusion: Our results suggest that the outcome of thermal cancer therapy is dependent on the temperature and the grade of fibrosis in the treated livers.*

Hepatocellular carcinoma (HCC) accounts for 85% to 90% of primary liver tumours and is the fifth most common malignant tumour in human (1). When a tumour develops in a cirrhotic liver, therapy modalities depend on tumour size, localization as well as staging and grading of the fibrosis or cirrhosis. Patients suffering from HCC are usually treated by liver transplantation or surgical resection (2, 3). In many cases, these options are not feasible due to the advanced stage of the liver tumour at the time of diagnosis. Alternative treatment options are transcatheter arterial chemoembolization, percutaneous ethanol injection therapy or thermal cancer therapy techniques, such as radiofrequency ablation (RFA), percutaneous microwave coagulation therapy and laser interstitial thermal therapy (4-9). These thermal techniques are minimally invasive and can be performed percutaneously. They are routinely used for treating HCC as well as for metastases of colorectal cancer (4, 10).

Independently of the source of energy, the effect of heat on the cell is strictly dependent on the temperature reached and the exposure time (7). The key aim for thermal therapy is achieving and maintaining a 60-100°C temperature range throughout the entire target volume in order to solely destroy the affected and not the healthy liver tissue (7, 11). Therefore it is essential to gain insights into the crucial parameters involved including blood flow and pressure (12-15) as well as the composition of the liver tissue (amount

of fibrosis or cirrhosis), and how these all influence the outcome of these thermal therapy techniques (16).

We know that the composition of liver tissue (*e.g.* the grade of fibrosis) is an important factor for the success of thermal cancer therapy (17). This study aimed to investigate the effect of hyperthermia on an HCC cell line (HepG2) and a hepatic stellate cell line (LX-1) to gain more information on thermal treatment of liver tumours. Different temperatures were applied to investigate the sensitivity of these different cell lines to heat injury. Furthermore, we documented the impact on heat exposure on different grades of simulated fibrosis to gather more information on thermal therapy in livers with different tissue compositions. These experiments are intended to provide data for planning thermal cancer therapy for patients suffering from HCC.

Materials and Methods

Cell culture and heating experiments. The human hepatocellular carcinoma 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 humidified atmosphere (5% CO₂). Hepatic stellate cells LX-1 (gratefully provided by Professor Friedman, Mount Sinai School of Medicine, NY, USA (18)) were cultivated in Dulbecco's modified eagle medium (DMEM containing 4.5 g/l D-glucose; Invitrogen, Austria) containing additional 10% FCS, 1% P/S and 1% L-glutamine (PAA) in a humidified atmosphere (5% CO₂). For all experiments, MEM with 10% FCS and 1% P/S was used.

Cells (3×10^4 cells/100 µl) were seeded in 96-well plates (#781962; Brand Tech Scientific Inc, Essex, CT, USA) and pre-incubated in MEM with 2.5 ng/ml recombinant human TGF-β1 (Biovision, Mountain View, CA, USA) for 18 h (18, 19). For the experiments, the culture medium was replaced by preheated medium. The plates were then incubated for different time spans (5, 10 or 15 min) in a heating cabinet preheated at different temperatures according to the experimental setup (55°C, 65°C, 75°C, 85°C). Thereafter, the medium was replaced by medium at 37°C. Metabolic activity was tested immediately after exposure to the different temperatures, at 24 h and 48 h after cell culture in culture medium at 37°C as mentioned below.

In order to simulate different grades of fibrosis co-cultures of HepG2 and LX-1 were cultivated at different compositions in one well (25% HepG2/75% LX-1, 50% HepG2/50% LX-1 and 75% HepG2/25% LX-1).

Metabolic activity testing. To test metabolic activity 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) was used as described by the manufacturer. After heat exposure, the hot medium was replaced by 100 µl medium at 37°C spiked with 20 µl MTS reagent. The absorbance of the soluble product is measured at 37°C at wavelengths of 490 nm (λ_1) and 650 nm (λ_2) using the SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA, USA). The metabolic activity was determined as the differences between absorbance of the two wavelengths ($\lambda_1 - \lambda_2$). The MTS test was performed immediately, and 24 h and 48 h after heat treatment.

Viability staining. Medium (500µl), containing 2×10^5 cells were seeded in 24-well plates and cultivated overnight. Thereafter the cells were exposed to different temperatures as described above. The Live/Dead® Viability/Cytotoxicity Kit (L-3224; Molecular Probes, Invitrogen, Austria) was used according to the manufacturer's protocol for staining. Fifteen minutes after heating, the cells were incubated for another 15 min at 37°C with 200 µl of the staining solution (containing 1 µl of Calcein AM and 4 µl ethidium homodimer-1 in 2 ml Phosphate Buffered Saline). Photodocumentation was performed with an inverted Olympus IX51 microscope. Alive cells exhibit in green fluorescence and dead cells stain red.

Statistical analysis. The mean of four different wells from each experiment was calculated and experiments were repeated 5 times. For each point of measurement, the data were normalized to a control value at 37°C. Statistical analysis and graphs were created with GraphPad Prism 5.00 software for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Comparison of the data was performed using one-way ANOVA with Tukey's post hoc test. A value of $p < 0.05$ was considered as being statistically significant.

Results

LX-1, HepG2 and their co-cultures resisted temperatures from 55°C to 75°C for 5 min with a metabolic activity of about 75% over 24 h and had nearly recovered completely after 48 h (Figure 1). After heating the cells for 5 min to 85°C the metabolic activity immediately decreased to 50% and to 10% over 48 h. Heat exposure for 10 min showed no permanent cell damage at 55°C (metabolic activity ~ 70%), whereas cell death was induced in almost all cells after 48 h of incubation after treatment at 65°C (~ 10%) and 75°C (~ 2%). Additionally, after 48 h still a metabolic activity of 40% was still measured after heating at 55°C for 15 min, whereas temperatures above 65°C led to complete cell death after 48 h of incubation.

The total metabolic activity decreased with a rising percentage of HepG2 cells in co-culture. Comparing the metabolic activity of LX-1 to HepG2 cells and their co-cultures clearly showed that LX-1 cells were more heat-resistant than HepG2 cells. The clearest differences were found after heat exposure to 55°C (Figures 2 and 3). Metabolic activity was significantly lower 24 h after heat exposure to 55°C for 5 min in 100% cultures of HepG2 cells compared to 100% cultures of LX-1 cells ($p<0.05$). After 48 h, the metabolic activity of 100% cultures of HepG2 cells was significantly lower than that of 100% cultures of LX-1 cells ($p<0.01$), and the cultures of 75% HepG2/25% LX-1 ($p<0.05$), 50% HepG2/50% LX-1 ($p<0.01$) and 25% HepG2/75% LX-1 ($p<0.01$).

Immediately after heating the cells for 10 min at 55°C 100% cultures of HepG2 cells had a significantly lower metabolic activity compared to 100% cultures of LX-1 cells ($p<0.01$), 25% HepG2/75% LX-1 ($p<0.05$), 50% HepG2/50% LX-1 ($p<0.001$) and 75% HepG2/25% LX-1 ($p<0.01$) cells. After 48 h but not after 24 h a similar significant heat tolerance pattern was observed. Metabolic activity of 100% cultures of HepG2 cells was significantly lower than those of 100% LX-1 ($p<0.01$), 75% HepG2/25% LX-1 ($p<0.05$), 50% HepG2/50% LX-1 ($p<0.01$) and 25% HepG2/75% LX-1 ($p<0.01$) cells. Immediately after heating for 15 min at 55°C, the metabolic activity of 100% cultures of HepG2 cells was significantly lower than 100% cultures of LX-1 ($p<0.001$), 50% HepG2/50% LX-1 ($p<0.01$) and 75% HepG2/25% LX-1 ($p<0.001$) cells. After 24 h significantly more viable 100% cultures of LX-1 ($p<0.01$), 25% HepG2/75% LX-1 ($p<0.05$), 50% HepG2/50% LX-1 ($p<0.05$) and 25% HepG2/ 75% LX-1 ($p<0.05$) cells

than 100% HepG2 cells were measured. All other points of measurement at 55°C showed the same trend.

Discussion

Thermal cancer therapy techniques are a minimally invasive option compared to resection and transplantation for treating HCC, even in patients with reduced general health conditions and liver function. We know that the temperature tolerance of cells depends on the composition of liver tissue (*e.g.* the grade of fibrosis), among other factors (17). But the exact conditions necessary to induce complete death of cancer cells have not been studied extensively yet. Most hyperthermic studies deal with temperatures from 42-46°C to investigate heat shock and thermotolerance (20), but hyperthermic cancer therapy methods are performed using temperatures ranging from 55°C to 100°C (7). Obara *et al.* showed in a cell culture study investigating the heat sensitivity of three different HCC cell lines that insufficient RFA, which does not kill all tumour cells, may induce further malignant transformation of HCCs (21). Therefore, we feel it essential to investigate a model to simulate these conditions in our cell culture study.

O'Neill *et al.* created a three-state mathematical model of hyperthermic cell death after heating the human lung fibroblast cell line MRC-5 and HepG2 cells (22). We chose the hepatic stellate cell line LX-1 to create a more physiological model of liver fibrosis and HCC (23). In order to activate cells and stimulate collagen synthesis simulating the surroundings in a cirrhotic liver, the cells were incubated with 2.5 ng/ml TGF- β 1 for 18 h as described in the literature (19). The activation of the TGF- β 1-treated hepatic stellate cells led to a higher heat resistance of the LX-1 cells (data not shown). Thermal cancer therapy is always performed percutaneously in a patient's liver. To compensate for the lack of tissue like cell structure (as the cells grow in a monolayer), we used shorter temperature exposure for our experiment.

In our experiments we found that a temperature exposure of 85°C for 5 min, 75°C for 10 min or 65°C for 15 min is necessary to induce cell death in HepG2 and LX-1 cells. Furthermore our results showed that HepG2 cells were very sensitive to heat-induced cell death but their sensitivity decreased with rising percentages of LX-1 cells in the co-

culture (Figures 2 and 3). This was a consistent trend and supports the theory on a cellular level that different grades of fibrosis affect the outcome of thermal cancer therapy.

This study provides an insight into the influence of hyperthermia by simulating a thermal therapy model in cell culture. We showed that the higher the percentage of fibrosis (the greater the proportion of LX-1 cells) the more heat/greater temperature is necessary in order to induce cell death in the area of interest. Our cell culture experiment supports the notion that temperature and the grade of fibrosis in the treated liver influence the outcome of thermal cancer therapy.

Acknowledgements

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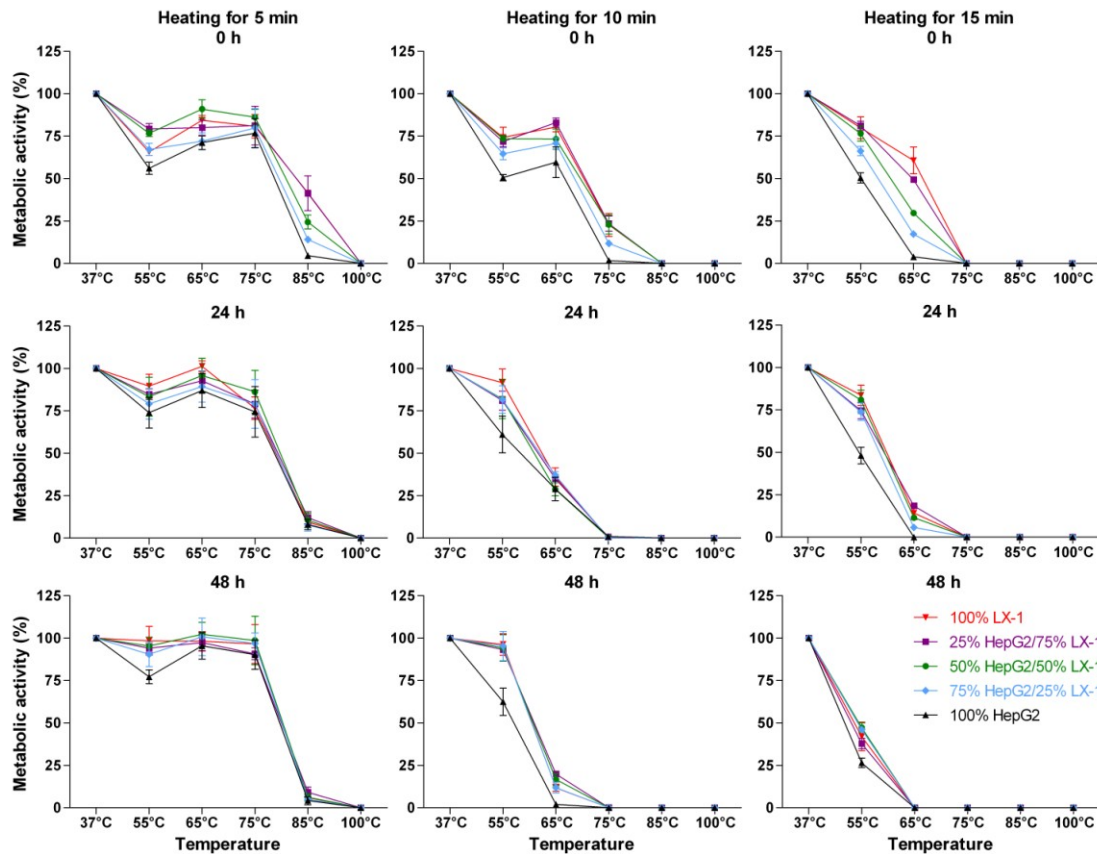


Figure 1. Shift of metabolic activity after hyperthermia of 5-15 minutes. Data were generated by heating the cell lines HepG2, LX-1 and their co-cultures at 55°C, 65°C, 75°C and 85°C. Metabolic activity was tested immediately after exposure to the different temperatures for different time spans (5, 10 or 15 minutes), after 24 h as well as after 48 h of cell culture. The data were determined using the MTS test and were normalized to the exposure at 37°C (100%). Our results showed that a temperature exposure at 85°C for 5 min, 75°C for 10 min and 65°C for 15 min leads to cell death in HepG2 and LX-1 cells.

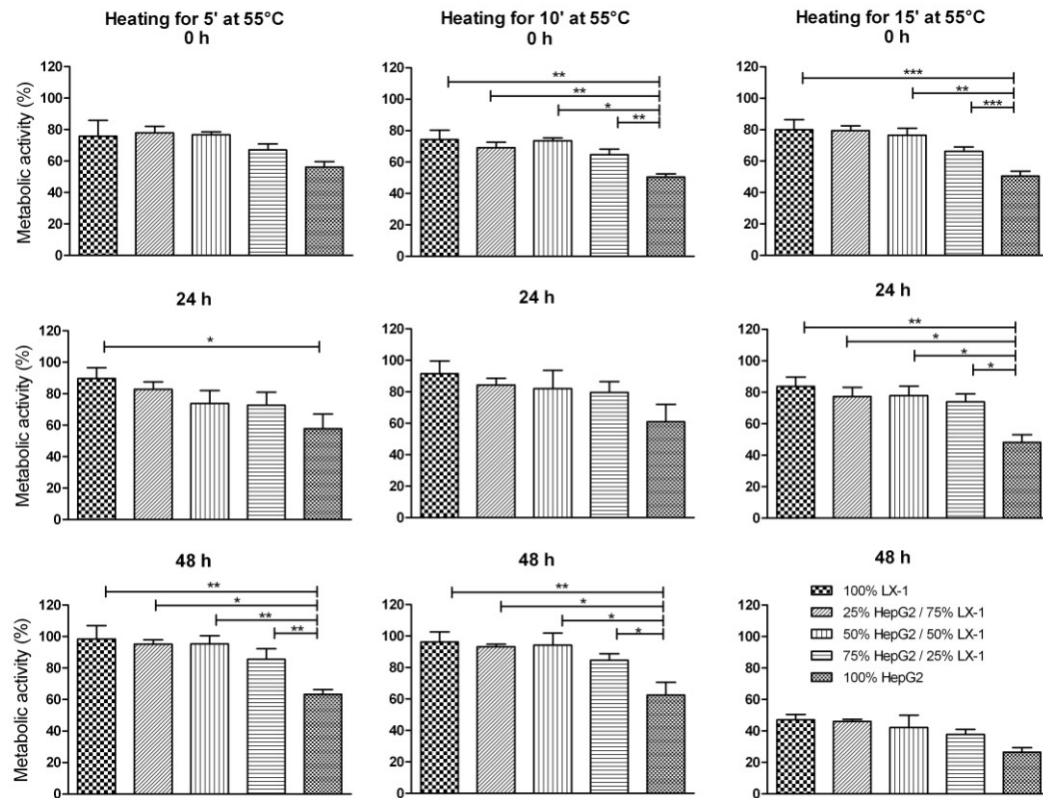


Figure 2. Significant differences in metabolic activity of liver cell lines and their co-cultures. Heating LX-1 cells, HepG2 cells and their co-cultures for 5 to 15 min at 55°C showed some significant differences in metabolic activity after hyperthermic treatment. Even where no significant significances were found, there was a trend for HepG2 cells being most sensitive to heat-induced cell death and their sensitivity decreased with rising percentages of LX-1 cells in the co-culture. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

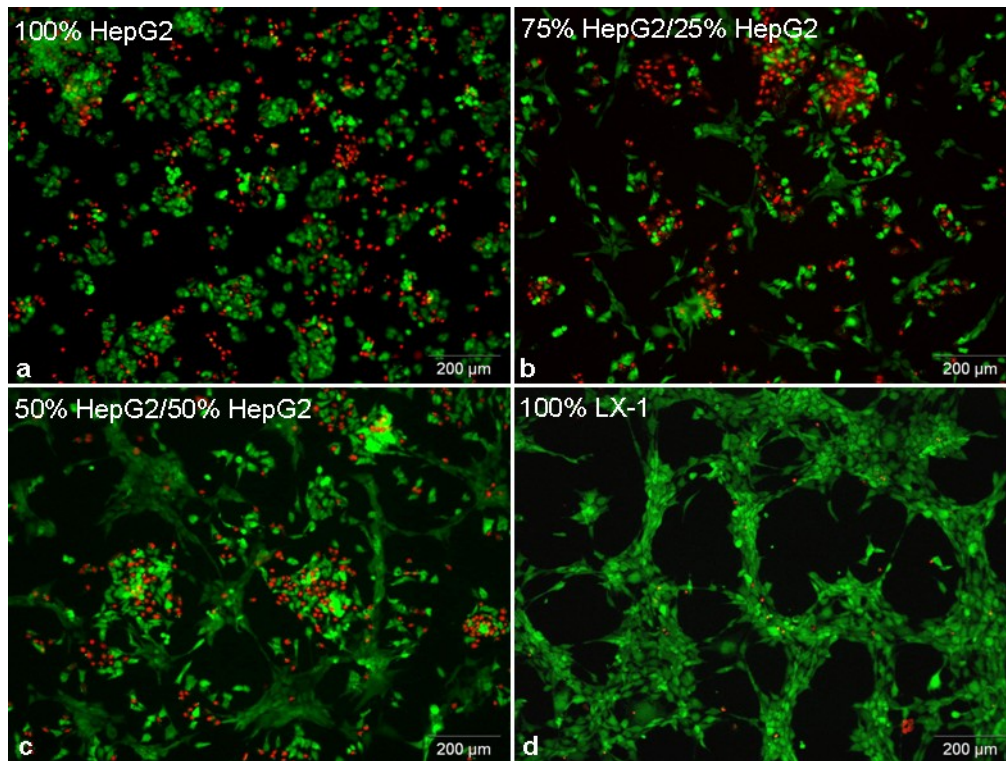


Figure 3. Differences in cell death induced via hyperthermia. Images showing differences in live and dead cell staining (Live/Dead® Viability/Cytotoxicity Kit) after heating LX-1, HepG2 and their co-cultures for 10 min at 65°C. HepG2 (a) are much more sensitive to hyperthermia than LX-1 cells (d). This was also shown in co-cultures, where metabolic activity decreased a rising percentage of HepG2 cells in co-culture (b, c). Green fluorescence: Viable cells; red fluorescence: dead cells. Magnification, x10.

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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). Material 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 higher a seeding density and more cell to cell interactions. Therefore, it is better 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.

Material 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 humidified atmosphere (5% CO₂). 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 medium (DMEM containing 4.5 g/l D-glucose; Invitrogen) containing additional 10% FBS, 1% P/S and 1% L-glutamine (PAA) in a humidified atmosphere (5% CO₂). For all experiments, MEM with 10% FBS and 1% P/S was used.

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%.

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 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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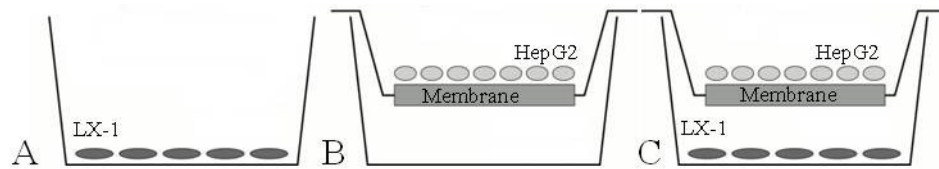


Figure 1. *Cells prepared for experiments: LX-1 cells were placed into the lower compartment and incubated with tumour growth factor- beta 1 for 18 hours overnight (A); HepG2 cells were grown on the membrane of the upper compartment (B). For the heating experiment and the follow -up culture, the cells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

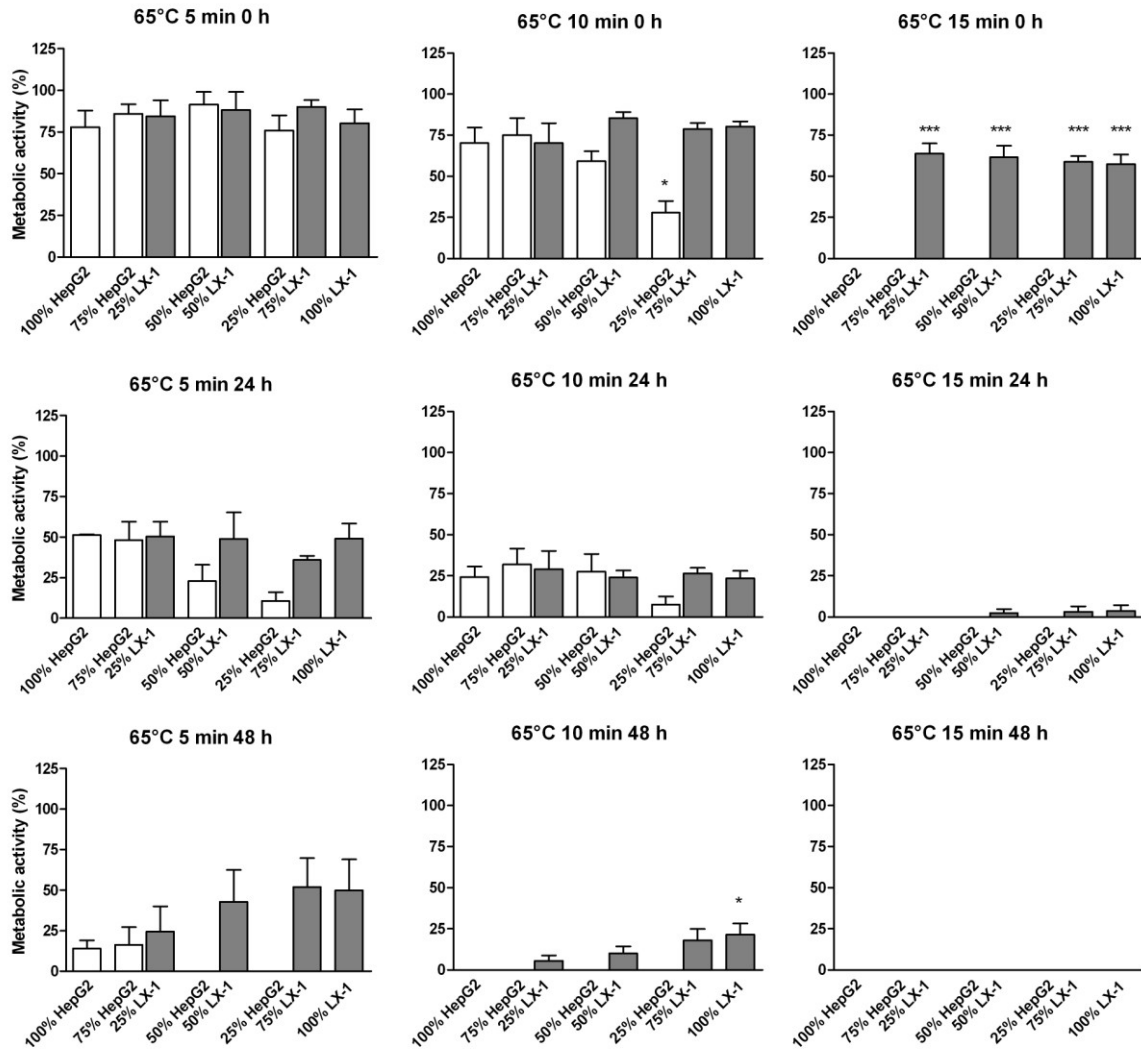


Figure 2. Metabolic activity of HepG2 and LX-1 cells after heating at 65°C in transwells. Our results showed that the higher the density of LX-1 cells in the transwells, the lower the metabolic activity of the HepG2 cells was. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc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

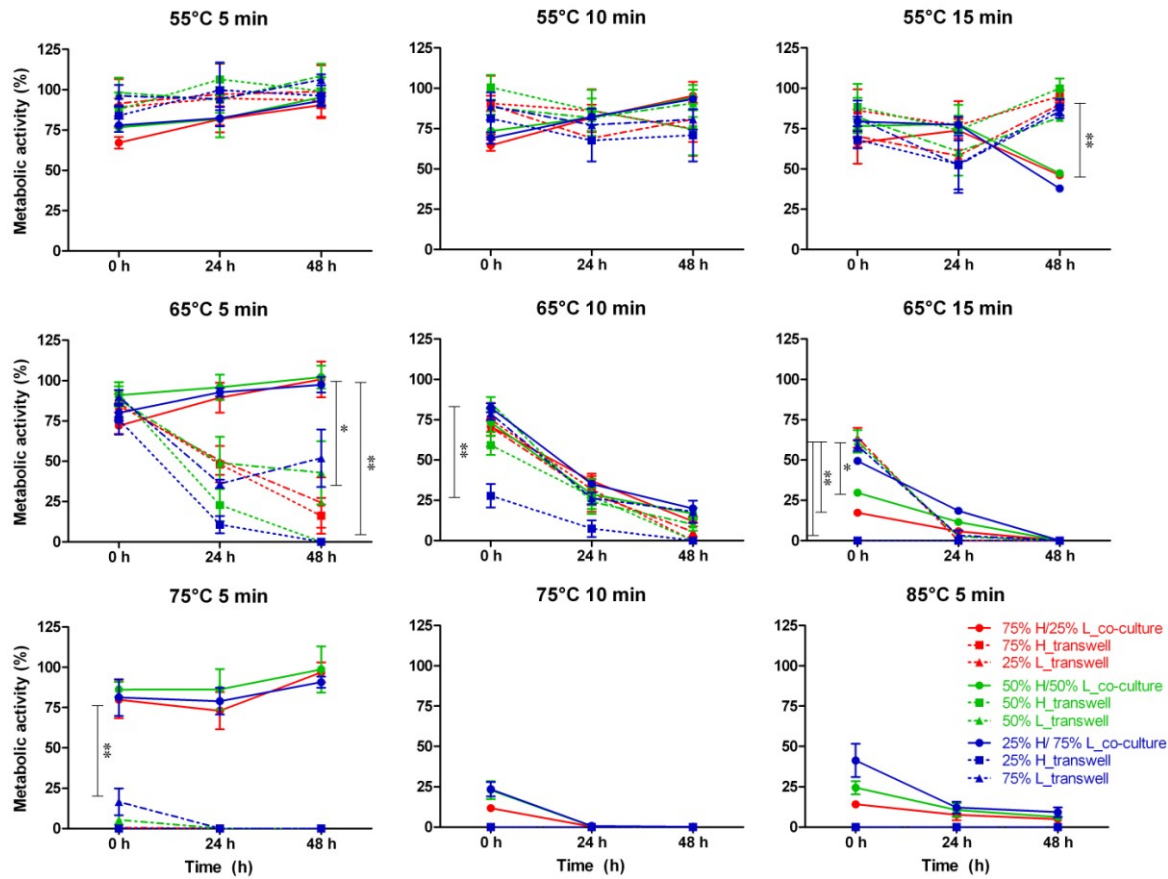


Figure 3. Comparing co-culture and transwell heating experiments. Heating HepG2 (H) and LX-1 (L) cells in co-cultures led to a better cell survival than cultivating them in transwells (* $p < 0.01$, ** $p < 0.001$).

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