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

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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. Materials 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.

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Key Words: Hyperthermia, thermal cancer therapy, hepatic stellate cells, fibrosis, HCC, HepG2.

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 humified atmosphere (5% CO_2). 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% FBS, 1% P/S and 1% L-glutamine (PAA) in a humified atmosphere (5% CO_2). For all experiments, MEM with 10% FBS and 1% P/S was used.

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 (λ1) and 650 nm (λ2) using the SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA, USA). The metabolic activity was determined as the differences between absorbance of the two wavelengths (λ1–λ2). The MTS test was performed immediately, and 24 h and 48 h after heat treatment.

Viability staining. Medium (500 μl), 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 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 be 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 similarly 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.

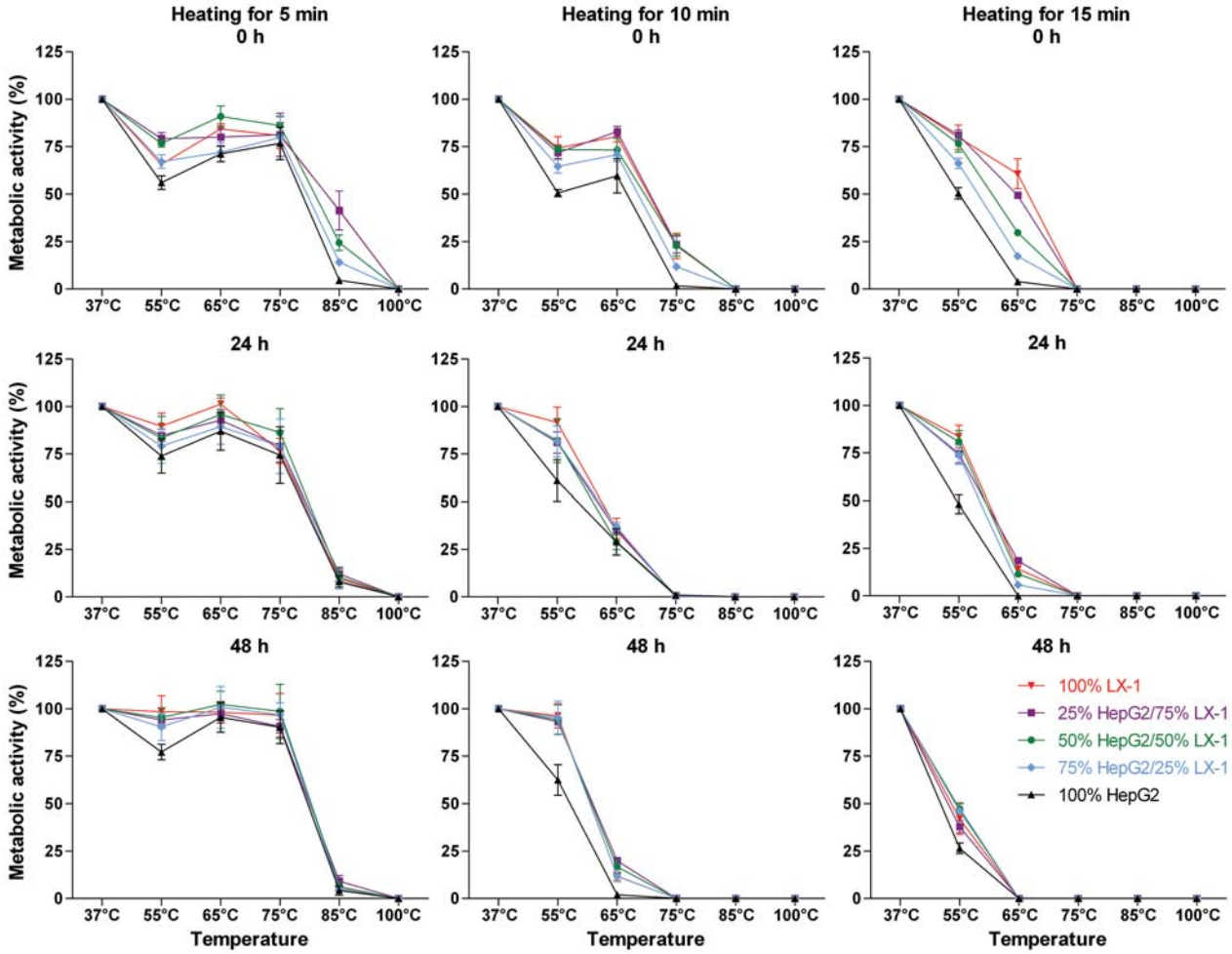

Figure 1. *Shift of metabolic activity after hyperthermia of 5-15 minutes. Data were generated by heating the cell lines HepG2, LX-1 and their co-cultures at 55˚C, 65˚C, 75˚C and 85˚C. Metabolic activity was tested immediately after exposure to the different temperatures for different time spans (5, 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.*

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-β1 for 18 h as described in the literature (19). The activation of the TGF-β1-treated hepatic stellate cells led to a higher heat resistance of the LX-1 cells (data

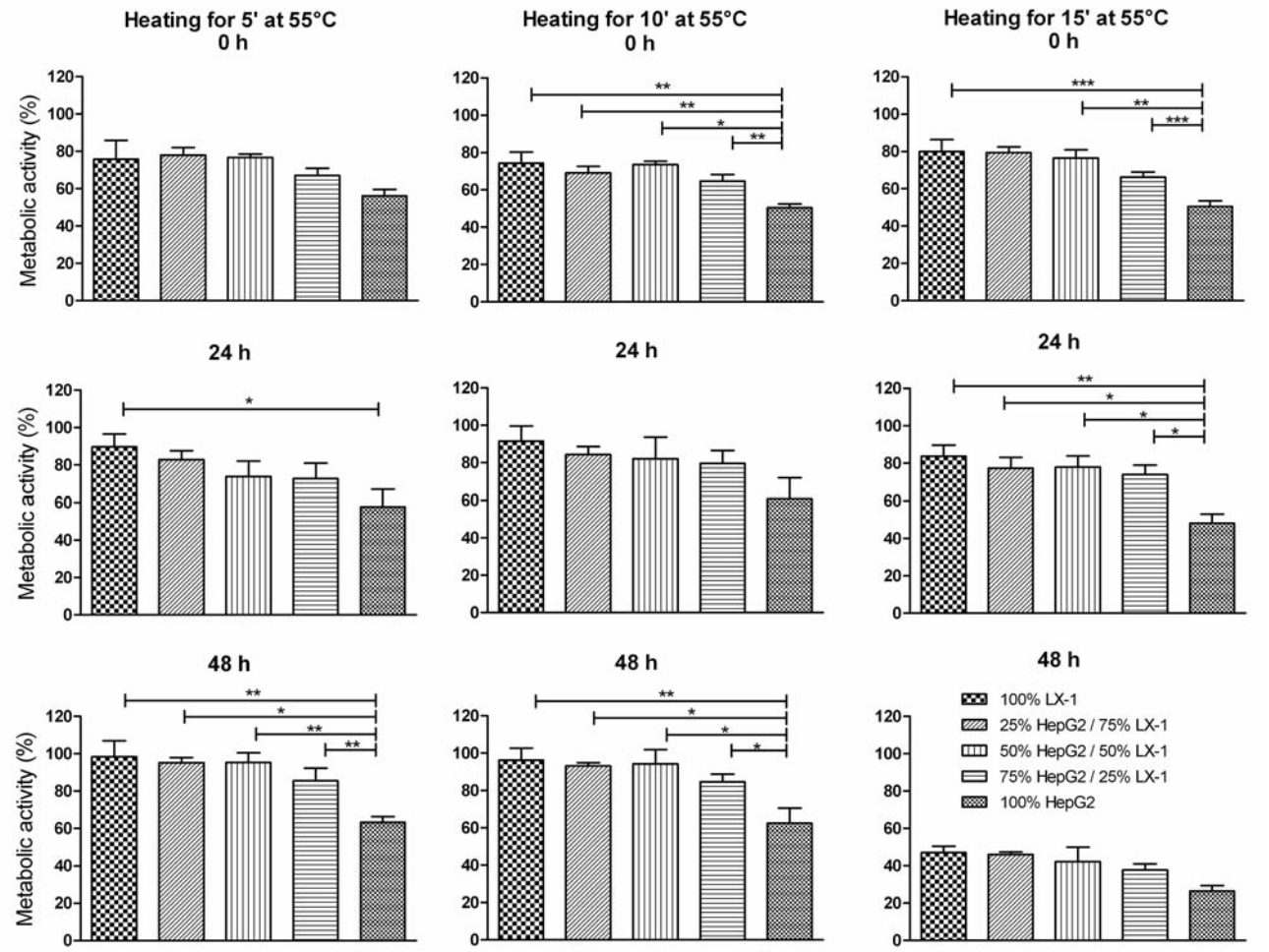

Figure 2. *Significant differences in metabolic activity of liver cell lines and their co-cultures. Heating LX-1 cells, HepG2 cells and their co-cultures for 5 to 15 min at 55˚C showed some significant differences in metabolic activity after hyperthermic treatment. Even where no significant significances 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).*

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

We wish to thank the Team of the Centre for Medical Research (ZMF) of the Medical University of Graz for excellent technical support. The research leading to these results has received funding from the European Community's Seventh Framework Programme under grant agreement no. 223877, project IMPPACT.

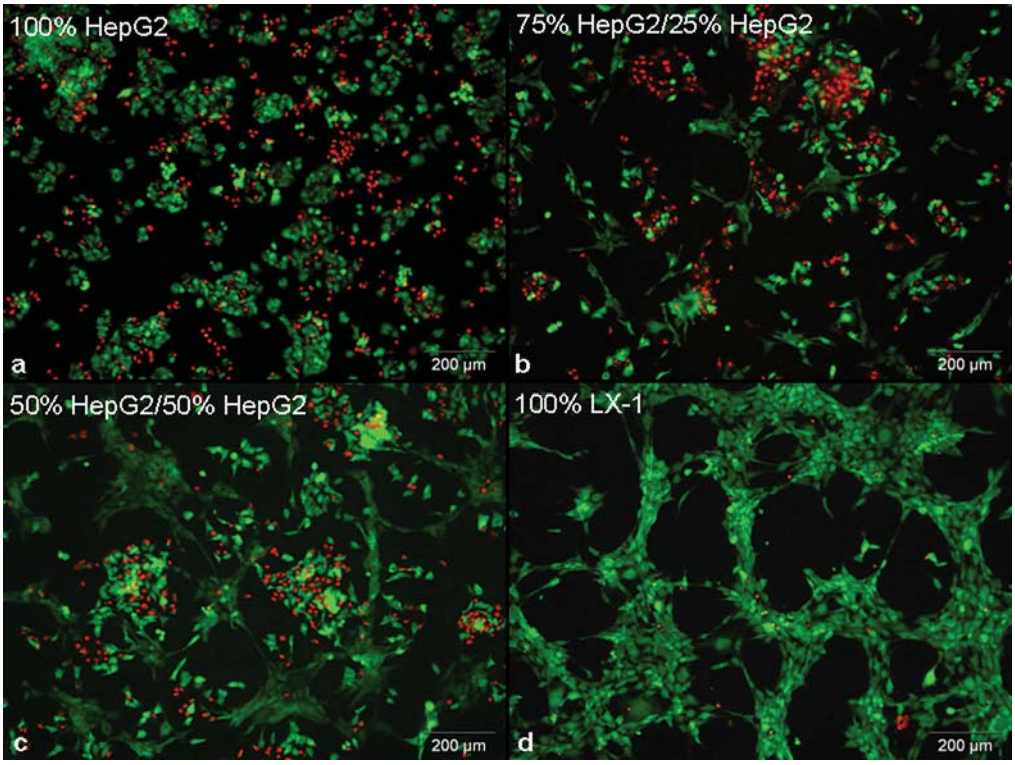

Figure 3. *Differences in cell death induced via hyperthermia. Images showing differences in live and dead cell staining (Live/Dead® Viability/Cytotoxicity Kit) after heating LX-1, HepG2 and their co-cultures for 10 min at 65˚C. HepG2 (a) are much more sensitive to hyperthermia than LX-1 cells (d). This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

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Received March 7, 2011
Revised April 18, 2011
Accepted April 19, 2011