

# Combined Hyperthermia and Radiation Therapy for Treatment of Hepatocellular Carcinoma

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## Abstract

**Background:** There is no doubt that hyperthermia is one of the powerful radiosensitizers. Finding a proper mechanism working in hyperthermia/radiation combination is still pronounced challenge. **Objectives:** This study is focusing on the anti-cancer activities (anti-proliferative, anti-angiogenic and antiapoptotic) of thermoradiotherapy. **Materials and Methods:** Liver cancer cell line (*HepG2*) was treated by 37°C, 40°C and 43°C hyperthermia degrees combined with three radiation doses (2 Gy, 4 Gy and 8 Gy) for 24, 48 and 72 hrs. Cell viability, apoptotic/necrotic cell screening, apoptotic (*BAX* and *FasL*) and antiapoptotic (*BCL-2* and *GRP78*) genes, and pro-angiogenic mediators [vascular endothelial- (*VEGF*) and Platelet derived-growth factors (*PDGF*) were investigated. **Results:** Our data showed that 40°C temperature combined with 4 Gy radiation gives a significant decrease ( $p < 0.05$ ) in cell viability. Maximum cytotoxicity was reported 48 hr post-treatment followed by slight restoration of cell viability after 72 hr. Compared with untreated cells, only 5% of viable cells with a high percentage of apoptotic (31%) and necrotic (63%) cells were demonstrated in 40°C/4 Gy/48 hr group. Expression of pro-apoptotic genes (*BAX* and *FasL*) were increased after hyperthermia with apparent elevation in 40°C/4 Gy/48 hr group coincides with moderate expression of antiapoptotic *BCL-2* and *GRP78* genes. A significant reduction ( $p < 0.001$ ;  $p < 0.05$ ) in *VEGF* and *PDGF* levels; respectively was shown at 40°C/4 Gy/48 hr group. **Conclusions:** This pilot study proposed 40°C mild temperature hyperthermia as a favorable hyperthermal condition with 4 Gy radiotherapy in HCC treatment. A further research has to be performed considering an application of more than one session of radiothermal therapy at 40°C/4 Gy for total abrogation of cancer cells.

**Keywords:** Hyperthermia- radiotherapy- cytotoxicity- angiogenesis- apoptosis

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## Introduction

Primary liver cancer is one of the predominant malignancies and represents a major health problem in developing countries. More than 90% of cases are hepatocellular carcinoma (HCC) (El-Serag and Kanwal, 2014). HCC is the sixth most common cancer worldwide, being the fifth in men and the eighth in women. It accounts for approximately 5.7% of all new cancer cases. Annually, around 1% of all deaths in the world were related to HCC. Despite recent discoveries in screening strategies and novel therapeutic options, HCC is still the third leading cause of cancer mortality worldwide and remains one of the most difficult tumors to treat (Samonakis and Kouroumalis, 2017).

Hepatic resection and transplantation have been considered as the essential curative therapies for HCC. Unfortunately, most of patients were surgically unresectable due to tumor size, location, or underlying

parenchymal disease (Llovet et al., 2015) Furthermore, the advanced neoplastic stage, severity of liver diseases or shortage lack of donors, restrict their application. For those cases, non-surgical therapies as chemotherapy and radiotherapy are recommended (Samonakis and Kouroumalis, 2017).

In the past few years, hyperthermia has been introduced as an effective approach for cancer therapy. The term 'hyperthermia' refers to several ways of heat application used as an adjuvant to establish strategy for cancer treatment such as radiotherapy and chemotherapy (Hildebrandt et al., 2002). With low side effects than chemotherapy and radiotherapy, adjuvant heat treatment has shown strong evidence in the management of tumors (Kampinga and Dikomey, 2001). Hyperthermia has been documented to have a killing effect on both normal and cancerous cells, even though; cancer cells are more sensitive to hyperthermia than normal ones (Kim et al., 2017). Efficacy of all hyperthermia modalities is not enough

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to replace any one of the established therapy modalities when applied alone, but, undoubtedly, they are suitable enough to enhance the cell-killing effect of cytotoxic drugs and/or radiation ('thermal chemosensitization', 'thermal radiosensitization') (Hildebrandt et al., 2002).

Several recent publications have focused on the effect of hyperthermia on distinct cellular signaling pathways, particularly of those involved in heat shock response, cell cycle regulation, and apoptosis. Moreover, hyperthermia influences tumor blood flow and oxygen/nutrient supply (Toraya-Brown and Fiering, 2014). Although there is a great number of researches on the therapeutic role of hyperthermia, there is little certainty about its effect on the process of angiogenesis (the sprouting of new capillaries from preexisting ones), which plays a vital role in tumor formation and maintenance (Bao et al., 2006). In the present study, we investigated the cytotoxic effect of combined therapy (thermal-radiosensitization) on HepG2 cancer cells and to clarify the role of this therapy on angiogenesis and apoptosis.

## Materials and Methods

### Cell culture

HepG2 cells were obtained from the American Type Culture Collection (ATCC) and were cultured at 37°C in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (200 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and HEPES buffer (1M) (All from Biowest, Nuaille, France). Cells were maintained in the logarithmic growth phase by routine sub culturing every 3–4 days in T75 tissue culture flasks. When reaching confluence, monolayer cells were rinsed with phosphate buffered saline (PBS) and harvested by trypsin/EDTA (Biowest) treatment. For the experiments, the cells were cultivated to 75% confluence on T25 tissue culture flasks.

### Combined (Thermal/radiation) treatment of HepG2 cells

Hyperthermia was carried out for 30min using 3 different incubators. After heating at 37°C, 40°C and 43°C, flasks were irradiated by Cesium 137 radiation source in the Egyptian Atomic Energy Authority (EAEA), with dose rate of 0.00751 G/sec. Thereafter, flasks were incubated for 24, 48 and 72 hrs. Time interval between thermal and radiation therapy is 5 min. Treated cells were grouped by temperature, radiation dose and length of incubation after treatment, generating 3×3×3 = 27 groups. Each group is consisted of 3 flasks and named by Temperature (37°C, 40°C, or 43°C)/Radiation dose (2G, 4G and 8G) and incubation period (24, 48 and 72hr) e.g. 37°C/2G/24 hr group. Blank wells consisted of cells cultured alone with complete medium.

### Cytotoxicity assay

Cell viability for all groups were measured by (3-(4,5-Dimethylthiazol)-2,5-Diphenyltetrazolium bromide) MTT assay as previously described (Talaat et al. 2014). Cell suspension (200µl) adjusted at 5×10<sup>4</sup> cells/well were seeded in triplicates into flat-bottom 96- well tissue culture plates (Griener Labortechnik, Kremmunster,

Austria). After removing the upper supernatant liquid (For VEGF and PDGF measurement), MTT solution (5 mg/ml) was added to each well (40µl/well) and incubated for 4hr. Insoluble purple formazan crystals resulting from reduction of the yellow tetrazolium salt of MTT by mitochondrial dehydrogenases in metabolically active cells were dissolved by the addition of 160 µl/well of acidified isopropanol (0.04N HCl in absolute isopropanol) for 24 hr at 37°C. The absorbance at 570 nm was measured (FLUOstar OPTIMA; BMG Labtech GmbH, Offenburg, Germany). The data are expressed as the mean percentage of viable cells as compared to the respective control cultures. The percentage of relative viability was calculated using the following equation: (Mean absorbance of treated cells/mean absorbance of control cells) X100.

### Apoptosis and necrosis staining

The type of the cell death was investigated in all treated groups. Cells were examined for morphological features of apoptosis (chromatin condensation and fragmentation) and necrosis by fluorescence microscopy using acridine orange and ethidium bromide (AO/EB) uptake as described previously (Baskić et al., 2006; Mamoon et al., 2009 ). In brief, a mixture of 100 µg/ml acridine orange and 100µg/ml ethidium bromide was prepared in distilled water. One microliter of dye mixture was mixed with 9 µl of cell suspension and the cell uptake of the stain was monitored under a fluorescence microscope (Axio Imager. Z2, Carl Zeiss AS Kabelgaten Oslo Norge) using annexin V/propidium iodide versus acridin orange/ethidium bromide and the apoptotic, necrotic and viable cells were counted. Treated cells were quantified by fluorescence microscopy according to the following descriptions: normal nuclei (bright green chromatin with organized structure), apoptotic (yellow chromatin that is highly condensed or fragmented), or necrotic (bright orange chromatin with round nuclei). The apoptotic index (percentage of apoptotic (or necrotic) cells) was calculated as number of apoptotic (or necrotic) cells/total cells counted.

### Detection of apoptosis by reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from HepG cells was extracted with the TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Extracted RNAs were dissolved in RNase/DNase free water and quantified using a NanoDrop 2000c spectrophotometer (Thermo Fischer Scientific, USA). The isolated RNAs had an A260/A280 ratio of 1.6–1.8. To check the quality of extracted RNA, agarose gel electrophoresis was used. One microgram of RNA was reversely transcribed into cDNA followed by PCR using SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Thermo Fischer Scientific). *BAX*, *BCL-2*, *GRP78*, *FasL* and *β-actin* (Huang et al. 2011) were amplified by PCR with specific primer set (Table 1). Each cycle set used a denaturing temperature (94°C) for 60s, annealing temperature (55°C-58°C) for 90 s, and extension temperature (72°C) for 90 s for a total of 40 cycles for

each primer, except  $\beta$ -actin where it was 30 cycles. The PCR products were separated on a 2% agarose gel containing ethidium bromide (0.5 mg/ml), visualized, and photographed using a 3UV Benchtop transilluminator Gel Documentation System supplied with DOC-IT (MSA) program (Ultra-Violet Products Ltd., UK). Relative intensity of the specific gene expression was analyzed by Image J software version 1.5J (NIH, Bethesda, MD, USA). mRNA expression was presented as expression index, the ratio of each signal to the signal from the  $\beta$ -actin housekeeping gene.

#### Determination of VEGF and PDGF by sandwich enzyme-linked immunosorbent assay (ELISA)

Levels of VEGF and PDGF were quantified by ELISA using commercially available matched paired antibodies (R and D Systems Inc., Minneapolis, MN) as previously described (Talaat et al. 2014). The intensity of the developed color was measured by reading optical absorbance at 450nm using a microplate ELISA reader (FLUOstar OPTIMA). The ELISA reader-controlling software (softmax) calculates the data of raw absorbance value into a standard curve from which cytokine concentrations of unknown samples can be derived directly. Results were expressed as ng/ml. Duplicate measurements of each parameter were used to obtain the mean absorbance value.

#### Statistical analysis

Data were pooled from at least three independent experiments, and presented as mean with the corresponding SE. Differences between groups were analyzed using one-way analysis of variance. All the statistical analyses were performed with SPSS19.0 (SPSS, Inc., Chicago, IL).  $P < 0.05$  was considered statistically significant.

## Results

#### Effects of hyperthermia and radiotherapy on cell viability

To monitor the potential effect of combined therapy on the tumor growth, initially we studied the cell viability at 24, 48 and 72 hrs of HepG2 cells gathering treatment with radiotherapy (2, 4 and 8G) and hyperthermia (37°C, 40°C and 43°C) (Figure 1). We found that the 40°C temperature combined with 4G radiation displayed a

significant decrease ( $p < 0.05$ ) in viability at different time intervals compared with untreated control group. Length of post-treatment incubation period was one of the most influential factors. Cell cytotoxicity was maximized after 48hr incubation time. A restoration of cell viability was notified after 72 hr incubation period. Surprisingly, comparing different radiation doses revealed that 8G was the worst dose while 4G was the best. It appeared that the action of combined therapy was started after 24 hr, reaching maximum after 48hr and partially lost after 72 hr.

#### Effects of hyperthermia and radiotherapy on apoptosis and necrosis

To investigate whether the intact membrane of treated cells underwent apoptosis or not, we studied the type of cell death using acridine orange/ethidium bromide staining to distinguish between apoptotic, necrotic and viable cells. The counting indicated that 40°C temperature combined with 4G radiation evolved a significant reduction ( $p < 0.05$ ) in the percentage of viable cells (5%) with high percentage of both apoptotic (31%) and necrotic (63.3%) ones (Figure 2).

#### Effects of hyperthermia and radiotherapy on gene expression

The effect of combined therapy on the expression of some pro- (*BAX* and *FasL*) and anti- (*BCL-2* and *GRP78*) apoptotic genes was investigated. As shown in (Figure 3), the expression level of *BAX* gene was varied. While an increase in *BAX* expression with hyperthermia was observed in 2G and 4G treated groups after 24 and 48 hrs with maximum production at 40°C/4G/48 hr, a reduction in 8G group was observed. Elevation in 8G group was restricted to normo-thermal ones. At the 72 hr, a severe reduction of *BAX* expression in all tested groups was found. Concerning *FasL* results, elevation in *FasL* was detected in almost all tested groups. The highest level of *FasL* expression was shown at 37°C/4G/24 hr, 40°C/2G/24 hr, 40°C/4G/48 hr, 43°C/4G/48 hr and 43°C/4G/72 hr.

Gradual reduction in *BCL-2* expression level was observed in 2G and 4G with various hyperthermia groups at different time intervals (Figure 4). There was a remarkable elevation in *BCL-2* in cells treated with 8G after 24 and 48hrs. Severe reduction in *BCL-2* was found in 40°C/48hr for all radiotherapy doses (2G, 4G and 8G).

Table 1. List of Primers of Selected Apoptotic/Antiapoptotic Genes (Huang et al., 2011).

Gene	Primers	Annealing	Product Size
Bax	F: 5'-TCTGACGGCAACTTCAACTG-3'	58°C	188bp
	R: 5'-TTGAGGAGTCTCACCCAACC-3'		
FasL	F: 5'-CTCTGGAATGGGAAGACACC-3'	57°C	325bp
	R: 5'-ACCAGAGAGAGCTCAGATACG-3'		
Bcl2	F: 5'-TCCATGTCTTTGGACAACCA-3'	56°C	203bp
	R: 5'-CTCCACCAGTGTTCATCT-3'		
GRP78	F: 5'-GCTCTCGAATCCAAAG-3'	56°C	331bp
	R: 5'-TTTGTCAGGGGTCTTTCACC-3'		
B-Actin	F: 5'-GAGACCTTCAACACCCCAGCC-3'	55°C	205bp
	R: 5'-GGATCTTCATGAGGTAGTCAG-3'		

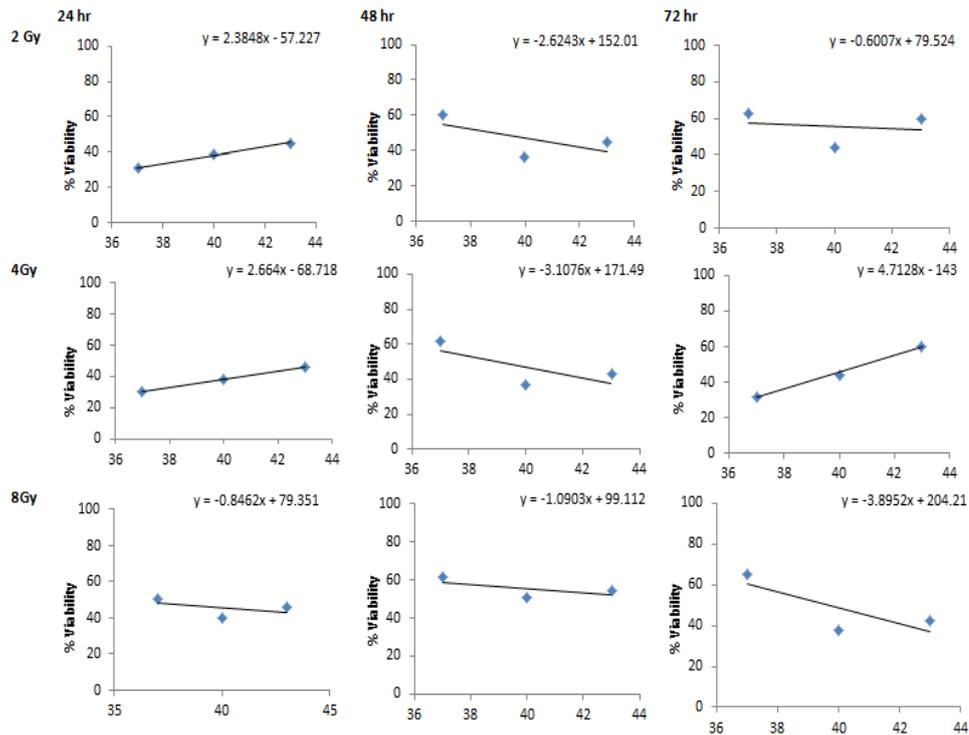


Figure 1. Cytotoxic Effect of Different Treated Groups on HepG2 Cells Using MTT Assay

Almost total abrogation of BCL-2 expression after 72hr at different groups of hyperthermia was observed in 4G and 8G groups. GRP78 expression level was increased in cells treated with 2G combined with 40°C at different time intervals followed by reduction in 43°C combinations. No change in GRP78 expression was observed in combination of 4G/8G with different hyperthermia degrees at various time intervals with only one exception with 24 hr which have very little amount of GRP78.

*Effects of hyperthermia and radiotherapy on pro-angiogenic factors*

As shown in Figure (5), insignificant change in VEGF

levels at different hyperthermia time or radiation doses after 24 hr incubation. A significant decrease ( $p < 0.001$ ) in production of VEGF was observed after 48 hr incubation in 2 treated groups; 43°C/2G and 40°C/4G as compared with normo-thermal group. In spite of temperature (37, 40 or 43°C), HCC cells subjected to 8G radiation produced the highest/stable amount of VEGF. Of surprising, dramatic reduction in the levels of VEGF for all combined treated samples at 72hr post-treatment was observed.

At certain radiation dose (2G and 4G), the production of PDGF was significantly ( $p < 0.05$ ) reduced by hyperthermia in all groups after 24 hr and 48hr incubation time in relation to normo-thermal group. As shown in

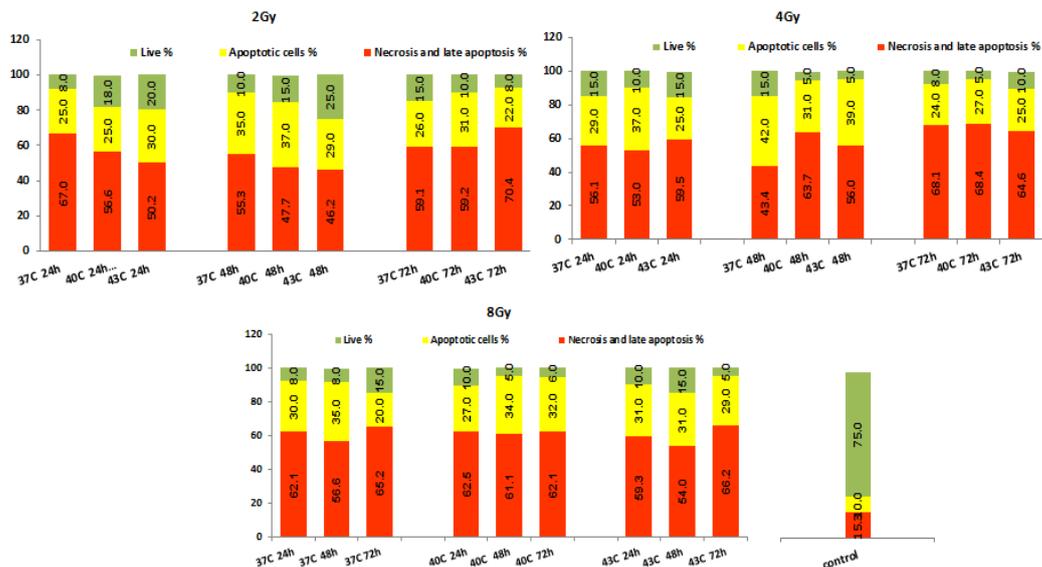


Figure 2. Comparison of the Number of Apoptotic/Necrotic Cells Scored by Fluorescence Microscopy Using Acridine Orange/Ethidium Bromide (AO/EB) in Different Treated Groups.

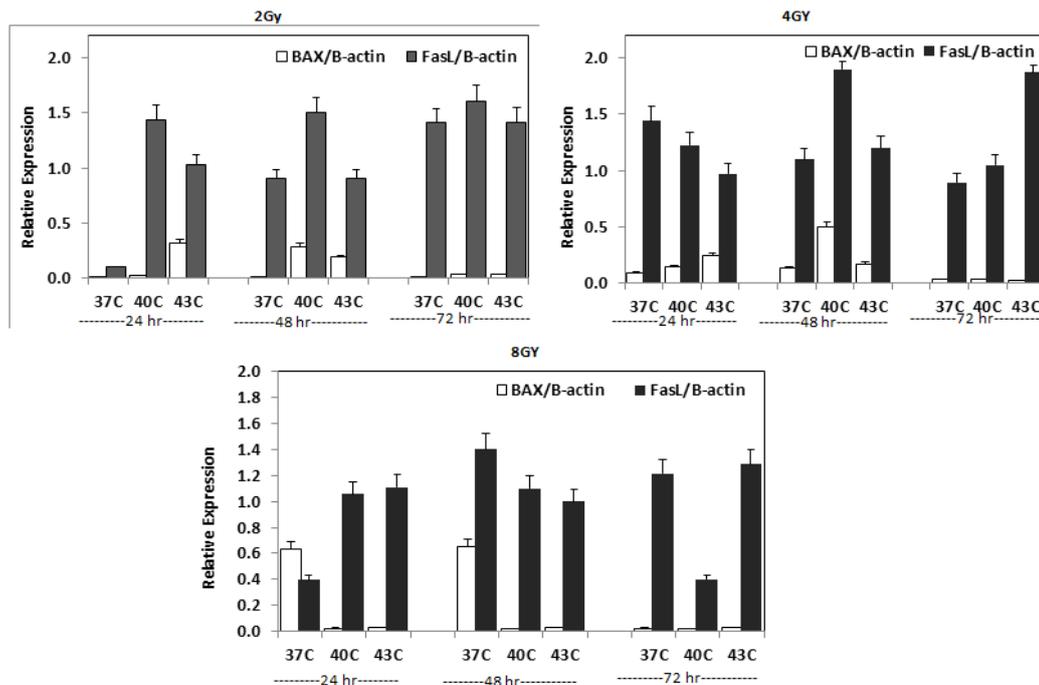


Figure 3. Apoptotic Gene Expression (BAX and FasL) in Different Treated Groups.

VEGF, the maximum reduction of PDGF was observed in 40°C/4G group after 48hr incubation. Although, PDGF was almost abrogated in 40°C/8G groups after 72 h, no significant change was demonstrated in the rest of groups.

### Discussion

The combination of hyperthermia with radiation has been discussed in several studies that show more achievement responses than radiation alone (Linchun et al., 2011; Dong and Wu, 2016). These studies with many others before have positioned hyperthermia as

the best radiosensitizer for treating tumors by radiation (Horsman and Overgaard, 2007). The clinical benefit of hyperthermia when combined with radiation has been proven in human trials for treatment of different types of malignancy including melanoma, glioblastoma, esophageal, head and neck, and cervix cancer (Chi et al., 2011). Regardless of these successes, the biologic rationale for combining hyperthermia with radiation is incompletely defined. Moreover, a significant variation in the response to hyperthermia between different types of cancers and/or tumors of the same type was demonstrated (Vaupel and Kelleher, 2010). In this work we have addressed an

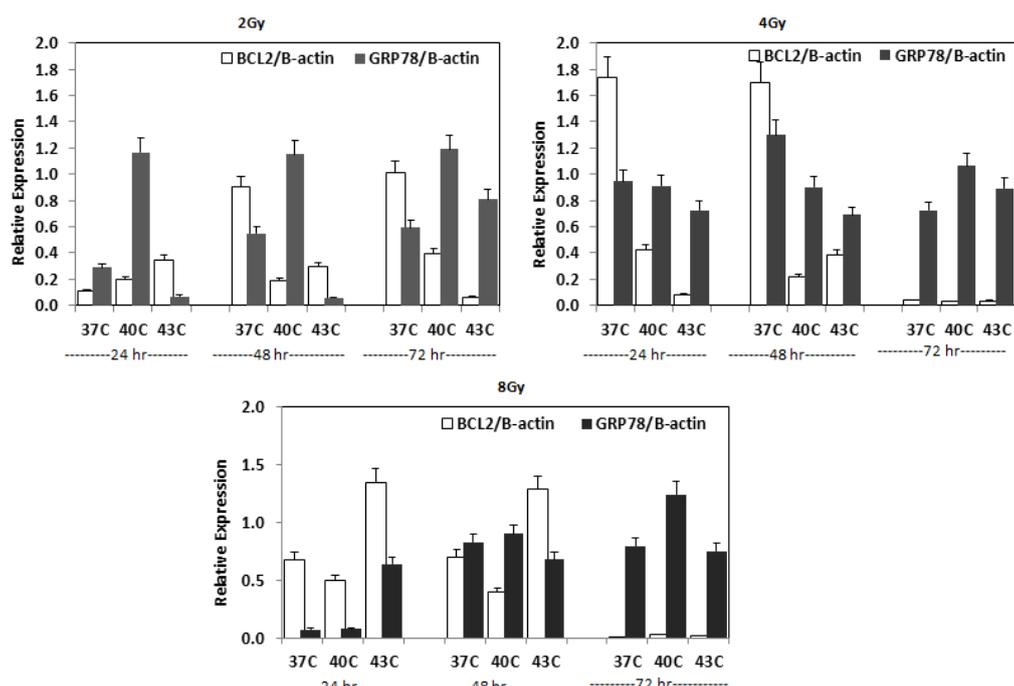


Figure 4. Anti-Apoptotic Gene Expression (BCL2 and GRP78) in the Different Treated Group

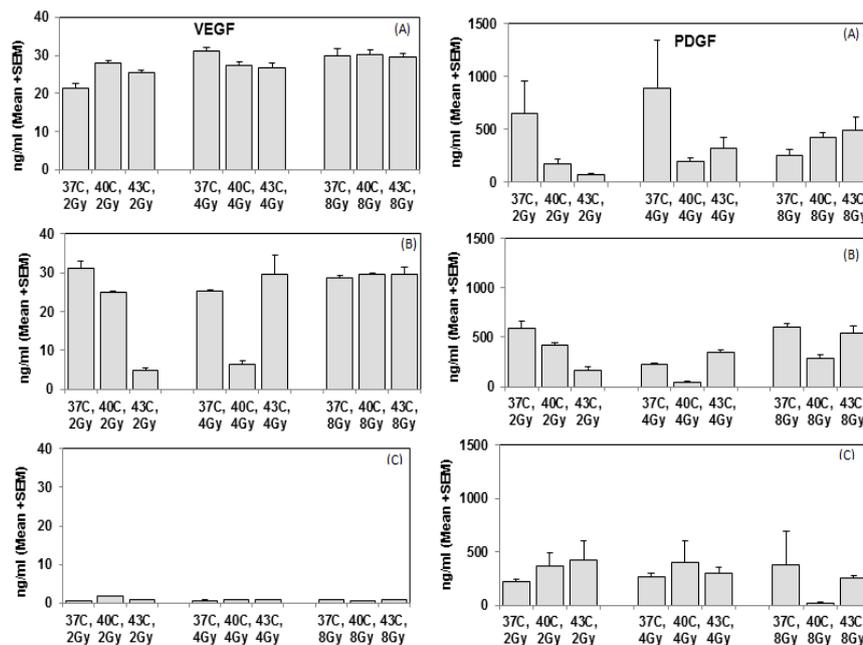


Figure 5. Level of VEGF and PDGF in Supernatant of Different Treated Groups. Results expressed as mean ± SEM

important question about the cytotoxic activity of thermal radiosensitization in treating HCC cells and possible mechanisms involved through studying the effect of temperature and time on angiogenesis in combination with apoptotic/antiapoptotic gene interventions.

Our results showed that, although the cell viability surprisingly increased with radiation (from 30% at 2G to 50% at 8G) in the normo-thermal groups (37°C), the combined treated samples reported more stable results. The 40°C combined with 4G notably had the best results for cytotoxicity (~60%). There is strong proof that the potential of complete response and thus tumor control is increased by adjuvant heat treatment. Evidences indicate that hyperthermia interferes with the cells' ability to deal with DNA damage after radiation. Temperatures which is higher than 42°C–43°C kill tumor cells directly and indirectly (Griffin et al. 2010). On the other hand, although mild temperature hyperthermia (MTH) (39°C–42°C) makes lower direct killing effects to tumors, it increases the cytotoxicity of radiation mainly by inhibiting the repairing mechanism of radiation-induced damage. This is consistent with our data where 40°C (MTH) has the maximum cytotoxicity effect. In vivo, the MTH; which also called the fever rang temperature; is effecting on immune-cells surrounding tumors that have an important role in cancer immunology (Takahashi et al., 2012). This appears clearly on natural killer (NK), dendritic and T cells, which increases its function at temperatures around 39°C–40°C and decline above that temperature (Ostbergi et al., 2003; Takahashi et al., 2012).

HCC tumors are highly vascularized, and the angiogenesis process is necessary for its development and progression (Tian et al. 2010). Among the angiogenesis factors, VEGF is the most important (Carmeliet, 2005) and its expression considered as a marker for HCC vasculature and metastasis (Kaseb et al., 2011). Following irradiation, angiogenesis and metastasis has been noted as an adverse effect of

radiation treatment in many cancer cells (Wild-Bode et al., 2001; Qian et al., 2002; Rofstad et al., 2004), with an uncertain clarity in the HCC recurrence mechanisms after radiation, the VEGF was over expressed after that treatment (Chung et al., 2006). In our result, VEGF and PDGF were temporarily expressed after 24hr and 48hr, before decline after 72 hr. The maximum reduction was notified after 48 hr with 43°C/2G and 40°C/4G treatment for both parameters. The PDGF has shown a similarity in variation of protein levels with VEGF which could indicate a common regulator in the HCC cells. Our findings of the relevance of change in expression of angiogenesis mediators are consistent with several other studies. The expressions of some genes associated with metastasis such as VEGF was shown to be suppressed by heating (Sawaji et al., 2002; Liang et al., 2010). In a previous study, Yuan et al., (2012) found that hyperthermia can inhibit hypoxia- induced Epithelial–mesenchymal transition (EMT) in HepG2 HCC cells and suggested that hyperthermia may alter the metastatic potential of cancer cells and inhibit tumor metastasis. The immunocytochemical localization of VEGF in cytoplasm and cell membrane demonstrated that the expression of VEGF in cells treated with hyperthermia for 30 min was significantly reduced compared with the control group. In accordance with our results, they detected a reduction in the expression of VEGF mRNA by hyperthermia when temperature was above 43°C (Xie et al. 2011). In another study, Kim and his colleges have shown that applying 41°C for 30 min. can reduce the radiation-induced upregulation of hypoxia inducing factor (HIF-1) and VEGF in FSaII fibrosarcoma inserted in legs of C3H mice (Kim et al., 2017). In contrast, other previous results showed that both radiation (Moeller et al. 2004) and hyperthermia (Moon et al., 2010) increase HIF-1 levels, which drives VEGF signaling.

At the molecular level, it was reported that apoptosis

is tightly regulated by caspases activation. Caspases can be activated through one of three pathways; extrinsic (or death receptor), intrinsic (mitochondrial) or endoplasmic reticulum (ER) pathways (O'Brien and Kirby, 2008; Huang et al., 2011). The extrinsic death receptor pathway is activated when death receptor of Fas (APO-1/ CD95) binds to its Fas ligand (FasL) resulted in receptor aggregation and recruitment of Fas associated death domain (FADD) (Hengartner, 2000). This recruitment leads to caspase-8 activation which directly initiates apoptosis by cleavage of caspase-3 (Nagata, 1997). The intrinsic pathway begins by targeting the mitochondrial permeability and cytochrome c release (Danial and Korsmeyer, 2004; Karpr, 2008). This pathway is regulated by *BCL-2* family proteins which classified according to their role in apoptosis to pro-apoptotic (e.g. *BAX*) and anti-apoptotic (e.g. *BCL-2*) proteins. Apoptosis initiation or inhibition is directed by pro- /anti-apoptotic proteins balance (Kroemer et al., 2007; Huang et al., 2011) ER can trigger apoptosis through the activation of the unfolded protein response (UPR), ER-resident cysteine protease, caspase-12, leading to caspase-3 activation and apoptosis (Zhang et al., 2006; O'Brien and Kirby, 2008). Glucose-regulated protein (GRP78), also referred to as BiP (immunoglobulin heavy-chain binding protein), is an endoplasmic reticulum (ER)-resident chaperon protein responsible for protein folding and assembly. It has its roles in activation of transmembrane ER stress proteins and the degradation of misfolded proteins (Lee, 2007). GRP78 is bonded with many of malignancies and human cancer cell lines including HepG2 (Jamora et al., 1996; Fernandez et al., 2000; Tchounwou et al., 2001; Croute et al., 2002).

Hyperthermia can initiate apoptosis by affecting its known three pathways (Ahmed et al., 2015). In our results, both extrinsic (*FasL*) and intrinsic (*BAX*, *BCL-2* and *GRP78*) pathways were investigated. Although the slight increase of *BCL-2* with hyperthermia at 24 hr, 2G group, the expression levels showing a decrease with hyperthermia at 4G/24 hr and 4G/48 hr groups. 40°C gives the lowest expression at 8G/24 hr and 8G/48 hr. After 72 hr post-treatment, the combined treatment has strongly decreased the expression with hyperthermia. Thus, our results indicate that *BCL-2* expression is down regulated by hyperthermia at 4G group in all three days. On the other hand, *BAX* expression is upregulated with hyperthermia at 4G group. In consistent to our results, Pagliari et al., (2005) showed that heating cells to 43°C can directly activate *BAX* and *Bak* to permeabilize the mitochondrial outer membrane to release cytochrome c. Moreover, the study of Liang et al., (2007), on effect of hyperthermia on human colon cancer cell line (HT29), indicated that the expression levels of *BAX* was up regulated with a contribution of down regulation in *BCL-2* levels and this effect exceeded when combined with radiation and chemotherapy.

The results of FasL expression pointed that at 4G, there was a slight decrease in FasL expression by hyperthermia, at 40 °C *FasL* was down regulated with high radiation doses at the three days. From these results, it seems that combination therapy affect the expression of *FasL*

negatively. *GRP78* expression pointed to a reduction in its level with hyperthermia and with radiation doses at 40°C. In agreement with our results, Xu et al., (2011) study indicated that *GRP78* exhibited an induction in its expression level on *AD293* kidney cell line subjected to 40°C and decreased by hyperthermia at 43°C.

In conclusion, our data focused on the importance of thermal-radio-sensitization as one of the effective anti-cancer therapy. We shed some light on 40°C mild temperature hyperthermia as the favorable hyperthermal condition with 4G radiation dose which affecting both angiogenesis and apoptosis processes. Our results stressed on the fact that the survived cancer cells after combined therapy can restore its efficiency and indicating to necessity for more than one therapy session. As delivering this fever range temperature to deep tumor malignancies as HCC is easy and applicable, this study may represent a major step in liver cancer treatment using radiothermal therapy.

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### Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

### Conflict of Interest Statement

None of the authors have any conflict of interest regarding the chemical agents or topics discussed.

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