RESEARCH COMMUNICATION

Killing Effects of Different Physical Factors on Extracorporeal HepG2 Human Hepatoma Cells

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Abstract

Objective: To determine the killing effects on extracorporeal HepG2 cells under different temperatures, pressures of permeability and lengths of treatment time. Method: According to different temperatures, pressures of permeability and lengths of treating time, extracorporeal HepG2 cells of human hepatoma cell-line were grouped to 80 groups. Cell index (CI) as the measurement of killing effect were calculated by monotetrazolium (MTT) methods, i.e., CI =1- (the OD value in treated group - the OD value in blank control group) / (mean of untreated control group – mean of blank control group). According to the factorial design, data were fed into SPSS 10.0 and analyzed by three-way ANOVA (analysis of variance). Result: Temperature, pressure of permeability and length of treating time all had effects on the CI (cell index) level. Length of treating time was the most influential factor of the three. Additionally, any two of them all had statistically significant interactive effects on the CI level. When treated for 5-30 min, destilled water at 46°C stably generated the highest CI. Conclusion: The "46°C-destilled water-60 min" was considered as the optimal combination of conditions which lead to highest CI. We suggest exerting celiac lavage for 15 min with stilled water at 40°C-43°C in surgical practice as a hyperthermia treatment to achieve ideal killing effects on free cancer cells, which is feasible, practical, and clinically effective.

Keywords: Killing effects - HepG2 cell-line - hyperthermia treatment - MTT method

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Introduction

90% cases of primary liver cancers are hepatocellular carcinoma (HCC) (Lopez, 2005). In that case, surgical resection is the preferented solution to liver cancers. However, the post-operation recrudescence is extremely high that the recrudescence rate within 5 year after simple resection is over 50%, among which most cases of metastasis outside liver are in abdominal cavity. First, the dropping and rooting of cancer cells during the surgical investigation and resection is an inevitable reality which significantly influences the recrudescence rate and sure rate. Once these free cancer cells (FCCs), which usually scatters on omental tissue, gastric membrane, intestine membrane, mesentery and peritoneal surface, got nutrition and blood supply, they would form tumors. Eliminating FCCs and tiny cancer nests remained in abdominal cavity is an essential part of post-operation health care. Second, one of the common complications of primary liver cancer is exploding spontaneous bleeding in hepatoma (Kim et al., 2009). The explosion usually results in a large quantity of cancer cells dropping in to abdominal cavity, i.e. FCCs, which became the seed of recrudescence (Huang et al., 2008). The discovery of method which can effectively kill FCCs in abdominal cavity will be truly significant in reducing recrudescence of hepatoma resulted by FCCs.

Hyperthermia has killing effect on both normal cells and cancer cells. However, cancer cells in anoxic environment are supposed to be more sensitive to hyperthermia than normal cells. Since tumors' reactions to hyperthermia varied largely according to their types (Yoo et al., 2006), it is worthwhile to investigate their sensitivity by type, in order to improve the effects of hyperthermia as while as to avoid damage to normal tissues as possible.

The cancer cell balances concentration electrolytical solution and pressure of permeability at both side of the cell membrane by transmitting substances through membrane. Changing pressure of permeability outside the cell would certainly change its internal environment, and in turn influence its function.

Our research was conducted in anoxic condition which imitates the environment of cancer cells to determine the killing effects to free HepG2 human hepatoma cells under different temperatures, pressures of permeability and lengths of treating time. It was aimed to discover a reliable and feasible method for killing FCCs during the surgery, in order to proactively prevent the post-operation recrudescence of hepatoma.

Materials and Methods

Instruments and materials

Human hepatoma cell line HepG2; Dulbecco's modified Eagle's medium (DMEM); Dimethyl Sulphoxide

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(DMSO); monotetrazolium (MTT); calf serum; constant-temperature water-bath tank (difference ≤ 0.1 °C); carbon dioxide (CO_2) incubator; culture medium; 96-hole cultivate plate, clean worktable.

Experiment method

Grouping

Affecting Factors: A. Temperature: 37 °C, 40 °C, 43 °C, 46 °C; B. Pressures of permeability: axenic distilled water; 0.45% axenic sodium chloride solution (hypostonic group), 0.9% axenic sodium chloride solution (isotonic group), 3% axenic sodium chloride solution (hypertionic group), and phosphate buffered saline solution (PBS group); C. Lengths of treating time: 5 min, 15 min, 30 min and 60 min.

Nomenclature: Each group is named by "Temperature-Pressure of permeability- Length of treating time", e.g., group 37-0.45%-5'.

Treated Groups: Based on a factorial design, the treated cells were grouped by temperature, pressure of permeability and length of treating, generating 4×5×4 = 80 groups. Each group included 6 samples (n=6).

Control Groups: Untreated cells in solutions are considered as the negative control groups, while cultivating solution without cells is the blank control group. Each of the two control groups included 12 samples.

Operation

Cell Cultivation: HepG2 cells were cultivated in DMEM (with calf serum, including PG100 U/ml, streptomycin100 μg/ml), placed in single-layer 50 ml/L CO_2 incubator with a constant temperature at 37 °C. Cells cultivated for three days, i.e. cancer cells at the exponential reproduction stage, were randomly sampled for the experiment. Sampled cells were made into suspension (with a concentration of 5×10^6 per micro liter, counted on tally). The suspension was distributed into sterile EP 2ml tubes of each group, 100 μl per tube, for consequent treatment.

Adding Solution: According to the grouping criteria, 900 μl 0.45% axenic sodium chloride solution, 0.9% axenic sodium chloride solution, 3% axenic sodium chloride solution, and phosphate buffered saline (PBS) solution, at 37 °C were respectively added to the previously prepared EP 2ml tubes of each group to reach a quantity of 2 ml per tube.

Temperature Control: We use water-bath to heat, i.e. constant-temperature water-bath tank (difference ≤ ±0.1 °C), and the tubes with different mixture were water bathed respectively at 37 °C, 40 °C, 43 °C, and 46 °C, for 5 min, 15 min, 30 min, and 60 min.

Measuring and Calculating CI by MTT Method: 200 μl treated cell suspension (5×10^5 per micro liter) were planted onto the hole of the 96-hole cultivate plate, i.e., 1×10^5 cells per hole. Each tube of cell suspension had to fill three duplicate holes. The untreated cell suspension was set as control holes (i.e. 200μl cell suspension per hole). Blank culture media without cells was used as the blank control group (i.e., 200μl per hole). After 50μl MTT was applied to each hole, mixtures on the entire hole plate were cultivated for 4 hours. After removing the upper supernatant liquid, 100μl DMSO was added to each hole. After concussed for 5 min, the optical density (OD) of each hole was measured by Enzyme linked immunoassay apparatus. Data collected were fed into the formula: CI (cell index) = 1- (the OD value in treated hole - the OD value in blank control group) / (Mean of untreated control group – Mean of blank control group), calculating the CI.

Data Analysis and results

CIs were expressed in form of $\chi\pm s$ (χstands for the mean value, s stands for the standard deviation). Experimental data were fed in SPSS 10.0 and analyzed by three-way ANOVA. The significance level was set to $p<0.05$.

Results

Individual effect analysis (Table 1): temperature, pressure of permeability and length of treating time all had statistically significant effects on the CI level. Length of treating time was the most influential factor of the three.

Multiple factor analysis: Except 40 °Cand 43 °C, CI at one temperature significantly differed from that at another, ceteris paribus (Table 2); 46 °C results in the highest cell death rate (i.e. CI mean =0.80069340). Except 0.45% and 3% axenic sodium chloride solution, CI in one solution significantly differ that in another, ceteris paribus; stilled water results in the highest CI mean (0.86252620) (Table 3). CI at each length of treating time differs significantly,

Table 1. Tests of Between-subjects Effects

Dependent Variable: RATE

Source	Type III Sum of squares	df	Mean square	F	Sig.
Corrected Model	14.376[a]	79	0.182	43.194	0.000
Inercept	255.652	1	255.652	60681.90	0.000
TEMP	1.020	3	0.34	80.681	0.000
INTEN	2.341	4	0.585	138.931	0.000
TIME	8.031	3	2.677	635.422	0.000
TIME*INTEN	0.956	12	0.080	18.917	0.000
TEMP*TIME	0.287	9	0.032	7.578	0.000
INTEN*TIME	0.749	12	0.062	14.822	0.000
TEMP*INTEN*TIME	0.991	36	0.028	6.534	0.000
Error	1.685	400	0.004		
Total	271.713	480			
Corrected Total	16.061	479			

[a]R Squared=0.895 (Adjusted R Squared=0.874)

Table 2. Multiple Comparisons of Temp

LSD (I)TEMP	(J)TEMP	MEAN Difference (I-J)	Sig.	95% Confidence Interval Lower Bound	Upper Bound
37	40	-0.05766035*	0.000	-0.07413375	-0.04118695
	43	-0.04301096*	0.000	-0.05948436	-0.02653757
	46	-0.12808216*	0.000	-0.14455556	-0.11160876
40	37	0.05766035*	0.000	0.04118695	0.07413375
	43	0.01464939	0.081	-0.00182401	0.03112278
	46	-0.07042181*	0.000	-0.08689521	-0.05394841
43	37	0.04301096*	0.000	0.02653757	0.05948436
	40	-0.0146939	0.081	-0.03112278	0.00182401
	46	-0.08507119*	0.000	-0.10154459	-0.06859780
46	37	0.12808216*	0.000	0.11160876	0.14455556
	40	0.07042181*	0.000	0.05394841	0.08689521
	43	0.08507119*	0.000	0.06859780	0.10154459

*The mean difference is significant at the 0.05 level

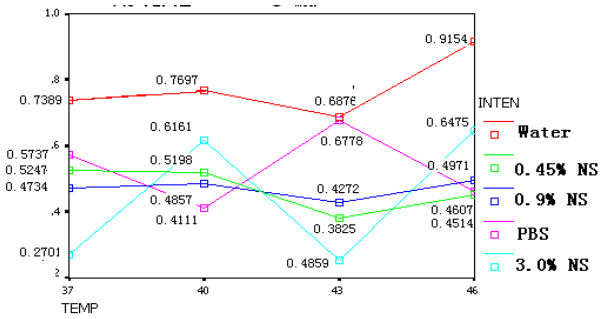

Figure 1. Estimated Marginal Means of CI at Time = 5 min

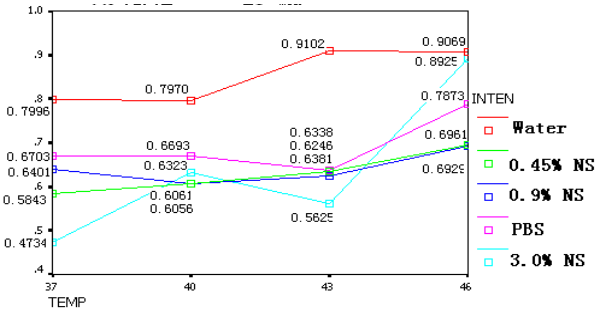

Figure 2. Estimated Marginal Means of CI at Time = 15 min

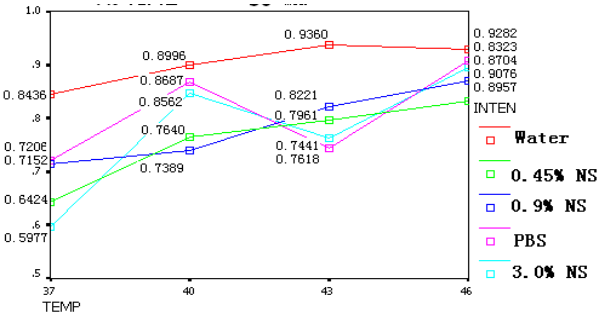

Figure 3. Estimated Marginal Means of CI at Time = 30 min

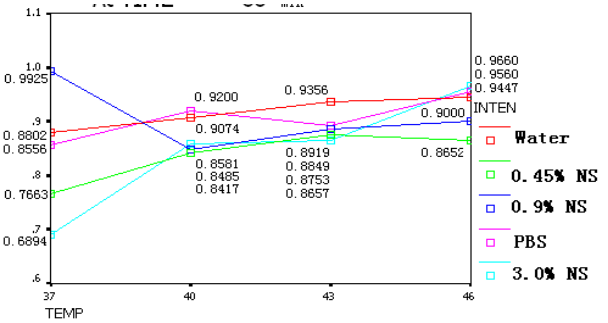

Figure 4. Estimated Marginal Means of CI at Time = 60 min

and increases as time progresses, ceteris paribus (Table 4). Thus, when factors are considered independently, the group of "46 °C-stilled water-60 min" is the optimal combination resulting in highest killing effect.

Interactive effect analysis: The interactions among these three factors (temperature, pressure of permeability and length of treating time) and between any two of the three are significant (Table 1).

Figure 1-4 clearly present the interactive effects among the three physical factors. According to the figures, the effect lines are unparallel, which implies the interactiveness among affecting factors.

Table 3. Multiple Comparisons of Inten

LSD (I)INTEN	(J)INTEN	MEAN Difference (I-J)	Sig.	95% Confidence Interval Lower Bound	Upper Bound
0.00	0.45	0.18865469*	0.000	0.17023687	0.20707251
	0.90	0.16132816*	0.000	0.14291034	0.17974597
	2.00	0.12797898*	0.000	0.10956116	0.14639680
	3.00	0.18567112*	0.000	0.16725330	0.20408894
0.45	0.00	-0.18865469*	0.000	-0.20707251	-0.17023687
	0.90	-0.02732653*	0.004	-0.04574435	-0.00890871
	2.00	-0.06067571*	0.000	-0.07909353	-0.04225789
	3.00	-0.00298357	0.750	-0.02140139	-0.01543425
0.90	0.00	-0.16132816*	0.000	-0.17974597	-0.14291034
	0.45	0.02732653*	0.004	0.00890871	0.04574435
	2.00	-0.03334918*	0.000	-0.05176700	-0.01493136
	3.00	0.02434296*	0.010	0.00592514	0.04276078
PBS	0.00	-0.12797898*	0.000	-0.14639680	-0.10956116
	0.45	0.06067571*	0.000	0.04225789	0.07909353
	0.90	0.03334918*	0.000	0.01493136	0.05176700
	3.00	0.05769214*	0.000	0.03927432	0.07610996
3.00	0.00	-0.18567112*	0.000	-0.20408894	-0.1672533
	0.45	0.00298357	0.750	0.01543425	0.02140139
	0.90	-0.02434296*	0.010	-0.04276078	-0.00592514
	2.00	-0.05769214*	0.000	-0.07610996	-0.03927432

*The mean difference is significant at the 0.05 level

Table 4. Multiple Comparisons of Time

LSD (I)TIME	(J)TIME	MEAN Difference (I-J)	Sig.	95% Confidence Interval Lower Bound	Upper Bound
5	15	-0.15186783*	0.000	-0.16834122	-0.13539443
	30	-0.26727245*	0.000	-0.28374585	-0.25079905
	60	-0.34297553*	0.000	-0.35944893	-0.32650214
15	5	0.15186783*	0.000	0.13539443	0.16834122
	30	-0.11540462*	0.000	-0.13187802	-0.09893122
	60	-0.19110771*	0.000	-0.20758111	-0.17463341
30	5	0.26727245*	0.000	0.25079905	0.28374585
	15	0.11540462*	0.000	0.09893122	0.13187802
	60	-0.07570309*	0.000	-0.09217648	-0.05922969
60	5	0.34297553*	0.000	0.32650214	0.35944893
	15	0.19110771*	0.000	0.17463341	0.20758111
	30	0.07570309*	0.000	0.05922969	0.09217648

*The mean difference is significant at the 0.05 level

The highest CI mean (0.99248400) appears in the "37 °C-0.9%-60 min" group, while the lowest CI mean (0.25483383) in "43 °C-3%-5 min" group. Highest CIs are those in the "46 °C, stilled water, 5 min." (0.9154), "43 °C-stilled water-15 min" (0.9102), and "43 °C-stilled water-30 min" (0.9360). However, there is a dramatic change when the treating time reaches 60 min: CIs in "46 °C-3%" group (0.9660) and "46 °C-PBS" group (0.9560) exceed that in the "46 °C-stilled water" group (0.9447). Therefore, treating for 15-30 min, the combination of "43 °C-stilled water" can stably result in the highest CI.

Discussion

Hyperthermia treatment whose rationale is based on cells' variant reactions toward hyperthermia attempts to kill cancer cell by heat. At present, induced apoptosis by hyperthermia has become a method to kill cancer cells; however, its mechanism has not been thoroughly understood (Hettinga et al., 1997; Ohtsubo et al., 2001; Shellman et al., 2008).

Though hyperthermia generally facilitates protein and DNA degeneration in cells, anoxic cancer cells were considered to be more sensitive to the hyperthermia than normal organic cells (Debes et al., 2002; Lim et al.,

2006; Dayanc et al., 2008). At the molecular biological level, hyperthermia effect catalyses the degeneration of protein in the cell membrane, and in turn reduces cells' self-stability. At the cellular level, hyperthermia facilitates apoptosis in S phase and M phase of cellular cycle. At the organic level, hyperthermia can cause microthrombus, oxidosis, hypoxia and dystrophy, thus, accelerates the degeneration and death of cancer cells. When at 43 °C, the hyperthermia can result in cell membrane protein reconstruction, handicaps in DNA reproduction, and deficiency in RNA and protein synthesis. These effects are intensified as temperature transcends 43 °C (Majima et al., 1992).

Other researches (Liu et al., 2002; Zhang et al., 2009; Zhao et al., 2010) show that, 42.5 °C is the critical point for maintenance of cellular function. Cancer cells usually started degeneration at 41 °C or above, and the degeneration would start accelerating at 43 °C. The cancer cells' idiosyncrasy, including hypoxia, nutrient deficiency, low pH value and low tolerance of high temperature, can further strengthen the killing effect of hyperthermia. Furthermore, the tumorous blood vessels differ from the normal vessels in that they were not dilatable under hyperthermia, thus the flux and velocity of flow would decrease apparently. Therefore the cancer tissues compared to the normal organs were more likely to have higher temperature under hyperthermia.

According to some researchers (Ding et al., 2005), hyperthermia at 43 °C for 30 min can generally lead to cell death in sensitive tissues, while when the hyperthermia is as high as 46 °C or above, necrosis occurs. Additionally, different cancer cells had variant reactiveness towards high temperature. Furthermore, the positive association between effects of hyperthermia risk of complications also increased the complexity. Generally speaking, the cautious selection of physical conditions to balance the beneficial effects and potential destruction is badly needed.

Our research experimented on free HepG2 human hepatoma cell, in an attempt to find a safe, effective and feasible way to conduct hyperthermia treatment, for reduction of the post-operation recrudescence in HCC. According to our ectogenetic experiment, our data suggest: (1) Temperature, pressure of permeability and length of treating time all had statistically significant effects on the CI level. Additionally, the interactions among these three factors and between any two of the three were significant as well. (2) Comprehensively considered, when treating 15-30 min interval the combination of "43 °C-stilled water" always led to the highest CI. (3) Length of treating time was the most influential factor of the three. (4) The interaction between these three factors beyond 60 min should be further researched to drawn reliable conclusions.

Thus, in conclusion, our results indicated that the group of "46 °C-stilled water-60 min" was considered as the optimal combination of conditions for highest CI, while We suggest exerting celiac lavage for 15 min with stilled water at 40 °C-43 °C in surgical practice to achieve ideal killing effects on FCCs. This practice is feasible and practical, thus clinically valuable.

However, our data had left some questions. When treating time was close to or exceed 60 min, "46 °C-3%" (CI mean=0.9660) and "46 °C-0.9%" (CI mean=0.9560) both led to slightly higher CI means than that of the stilled water group (CI mean=0.9447). The highest CI mean (0.99248400) even appeared in the "37 °C-0.9%-60 min", which greatly contrast with what happened in the 5 min, 15 min, and 30 min time point. Inferably, beyond 60 min, temperature and pressure of permeability's effects might be subdued, or other essential factors had come into play.

A possible explanation was that due to the prolonged hyperthermia treating, cancer cells' hypoxia and nutrient deficiency last for such a long time that cellular secretion and toxin had accumulated to a certain extent, which eventually lead to cell death. Thus, the actual interactive effect of "temperature-pressure of permeability-treating time" and its resulting killing mechanism beyond 60 min needed further exploration and experimentation. Nevertheless, the reverse fluctuation happened at 60 min implied the existence of an optimal combination of temperature, pressure of permeability and treating time, under which the killing effect to a certain type of cancer cells will be more efficient than that under other combination of the physical conditions. Secondly, in the reality, to avoid destructing normal organs, we could not infinitely increase the hyperthermia treatment in temperature and time.

Some research suggested that during the general anesthesia, though celiac lavage by warm (42°C~45°C) stilled water for 15 min would lead to the rise of body temperature, speeding-up of HR and drop of blood pressure, this short-term lavage was yet within the allowance of compensation of the body, which would not result in a decrease of blood perfusion of the tissue and hypoxia in the relevant organs. Nevertheless, if the temperature of lavage was too high, it would cause a sharp rise of body temperature, and lead to the dilatation of encephalic vessels, and rise of encephalic pressure. It was also suggested that ice bag can be employed to lower the brain temperature; however, no limit of time length was suggested.

According to the results of our research, treating for 15-30 min, the combination of "43 °C-stilled water" can stably result in the highest CI. And there was no statistically significant difference between effect of 43 °C and 40 °C groups, where CIs both increased significantly as treating time prolonged. Therefore, we contend that exerting celiac lavage for 15min with stilled water at 40 °C-43 °C in surgical practice, to achieve a good killing effects on FCCs.

Early diagnosis, early treatment is critical in therapeutic effect to liver cancers. Conducting celiac lavage hyperthermia treatment to kill FCCs, at the same time with thorough or partial resection, will be significant in reducing the recrudescence rate and mortality rate from HCC.

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