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Frequency Effect on Proliferation of Human Liver Cancer Cells under Alternating Magnetic Field

Do Guwn Hwang* and Hyeji Park

Department of Oriental Biomedical Engineering, Sangji University, Wonju 26339, Republic of Korea

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The effects of alternating magnetic field (AMF) stimulation on the proliferation of human liver cancer cells (HepG2) under varying frequencies from 500 Hz to 2 kHz were investigated. The AMF stimulator generated a magnetic field of 5 mT with a saw tooth pulse waveform, the frequency of alternating pulses was controlled by the duty factor of the digital control circuit. HepG2 cells were cultured in a 6-well plate over a magnetic coil using a cooling system while been kept for 48 hours in an incubator. After magnetic stimulation of 0.5, 1 and 2 kHz, the proliferation rates of the HepG2 cells saw almost no difference compared to liver cancer cells that were not stimulated. However, the proliferation rates at 1.4 kHz were reduced by up to 66 % compared to the non-stimulated cells. In other words, HepG2 cancer cells can be suppressed by magnetic stimulation of a certain frequency. These results are in good agreement with those from analysis of cell viability using MTT assay, a colorimetric assay used for assessing cell metabolic activity. This phenomenon of a suppression frequency may be related with various ionic flows that occur in the ion channels of cell membranes.

Keywords : alternating magnetic field, magnetic stimulation, liver cancer, HepG2, proliferation

1. Introduction

Alternating magnetic field (AMF) stimulation has been suggested as a useful tool for suppressing proliferation of cancer cells, it's main attraction is that this approach could affect the bioelectricity of ionic flow in the cellular membrane in a non-invasive manner. The influence of alternating magnetic field on the proliferation of cancer cells is being actively studied for frequencies near 60 Hz or looking at frequencies in the MHz and beyond. However, research looking at a range of intermediate frequencies in the kHz region with intensities measured in mT has not yet become common. This frequency range is very important because the ion exchange flows of K^+, Na^+, and Ca^+ in ionic channels have relaxation times on the order of milliseconds [1].

Most studies on the effect of magnetic field stimulation on cancer cells *in vivo* and *in vitro* have been performed at extremely low frequencies with electrical appliances using 50-60 Hz or have used radio frequencies of several MHz to GHz. Wang *et al.* studied cancer cell lines that were exposed to a low-frequency alternating magnetic field. They reported that exposure to a magnetic field of 0.4 Tesla at 7.5 Hz could suppress cancer cell proliferation and disturb the cell cycle [2, 3].

Human liver cancer cells (hepatocellular carcinoma) are one of the most common causes of cancer-related deaths, the worldwide incidence of this cancer has been increasing in recent years. Most hepatocellular carcinoma is not cured by conventional treatments, such as surgical ablation, chemotherapy or liver transplantation. For all stages combined, the relative 5-years survival rate from liver cancer is about 15 % [4].

Luciana Dini *et al.* reported that the viability of human liver cancer cells (HepG2) when exposed to a static magnetic field of 6 mT over 4 hours did not change, but the viability did decrease by about 20 % after 24 hours of exposure. The cell shape was extensively modified after 24 hours of exposure to a 6 mT field [5]. Dr. Rosen suggested that a static magnetic field can alter the function of trans-membrane flux through ionic channels [6]. Living cells are able to respond to electric and magnetic field stimulus, this is because this kind of stimulus affects the molecular structure of the excitable membrane and ion specific channels. Cellular responses depend not only on the intensity and frequency of the field, but also on the

*Corresponding author: Tel: +82-33-730-0413
Fax: +82-33-764-6710, e-mail: ndghwang@naver.com

type of field (static and oscillatory), on the waveform (sinusoidal, square, saw tooth) and on the biological status of the exposed cells [7].

Most studies has been preformed using stimulation conditions of a constant frequency and intensity, this is because it is hard to control changes to both frequency and intensity using the impedance mismatch of a magnetic coil. In order to understand the effect of magnetic fields on ionic flow in cellular membranes, it is vital that we control the frequency without changing the magnetic intensity while stimulating the living cells. Therefore, a well-designed stimulus system that avoids coil heating with increasing frequency is required [8].

For this work, we created an AMF stimulation system consisting of a switched-mode power supply and a digitally controlled circuit for modulating the duty ratio. The system generates a magnetic field with a saw tooth waveform up to 7 mT and 4 kHz without any coil heat. The effect of AMF stimulation on the proliferation of human liver carcinoma (HepG2) cells as a function of frequency from 500 Hz to 2 KHz at 5 mT was investigated.

2. Materials and Methods

Commercial AMF devices generate a magnetic field with a sinusoidal waveform in magnetic coil. These systems have two problems: increasing coil heat and decreasing field intensity as the frequency increases. This leads to the coil temperature needing to be reduced at high frequencies, because all cells in the incubator die will when the temperature passes 37 °C. Our device was designed to provide a pulse waveform in a saw tooth shape, as shown in Fig. 1(c), while maintaining a constant intensity of 5 mT as the frequency increases up to 4 kHz. The intermediate frequency range in the kHz region is related to the response time of certain ionic flows (10^{-3} sec) in cellular membranes, as such these flows can be excited by this kind of external stimulus. In our previous work, we reported that a sinusoidal AMF stimulator causes the coil temperature to increase rapidly compared to a stimulator using a saw tooth waveform [8].

The AMF stimulator consists of a switched-mode power supply and a digital circuit for controlling the duty ratio α/β, which is defined as the ratio of pulse width, α, over the time duration of pulses during a half cycle, β. In addition, the circuit of our system contains a four field transistor and insulated gate bipolar transistors, as shown in Fig. 1(a) and (b). Our stimulator can provide a maximum current of 40 A, and generates a magnetic field in the of a saw tooth pulse wave. The intensity of the field can be maintained at 5 mT for a frequency of 2 kHz without coil heat.

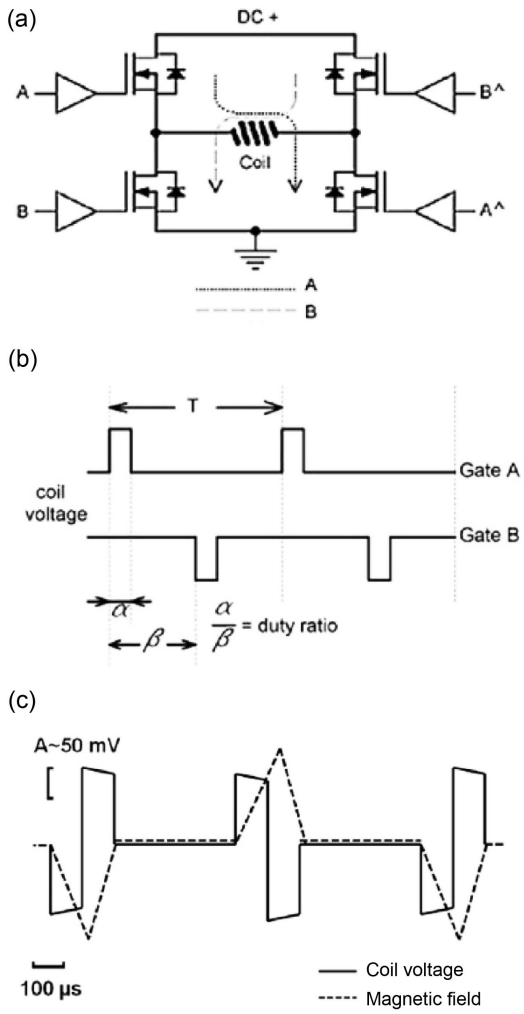

Fig. 1. (a) Schematic diagram of designed circuit with an insulated gate bipolar transistor (IGBT), (b) the duty ratio α/β was defined as the ratio of pulse width, α, to the time duration of the subsequent pulses over a half cycle, β (c) Real oscilloscope image of the applied voltage and the magnetic field recorded from the second coil at 1.2 kHz.

The temperature of the 6-well cell plate in incubator used in our experiments did not exceed 37 ± 1.0 °C during magnetic stimulation. The magnetic coil used consists of a single layer of 15 turns with a flat shape and dimensions of 14 × 6 cm, the cross section of coil wire is 1 mm thick and 3 mm wide. The 6-well plate of 12.7 × 8.5 cm was placed on the coil along with the water cooling system. The HepG2 cancer cells were then cultured over the magnetic coil and kept in the incubator for 48 hours. The cells were cultured in three plates at the same time, as shown in Fig. 2. One plate was cultured with no magnetic stimulation, the other two plates were cultured under magnetic field stimulation, two plates were used to increase

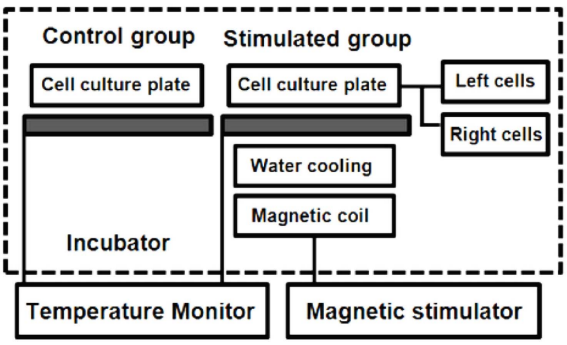

Fig. 2. Schematic diagram of AMF stimulator, magnetic coil, water cooling system, and cell culture plates.

the reliability of the data gathered.

We determined an exposure time to stimulation of 48 hours, this period was chosen because the number of HepG2 cells doubles from 1×10^5 to above 2×10^5 after 48 hour of proliferation. If the number of cells in the control group that experiences no magnetic stimulation was below 2×10^5, all these results were excluded from our statistical data. The cell groups under stimulation in the other two plates were cultured under fields of the same frequency and intensity for experimental reliability. If the difference in the number of cells between the two groups under stimulation was above 15 %, all these results were excluded from our statistical data.

Human liver HepG2 cancer cells were obtained from theKorean Cell Line Bank. The cells were cultured in the PRMI-1640 medium (Roswell Park Memorial Institute) with 5 % FBS (Fetal Bovine Serum) in a humidified atmosphere with 5 % CO_2 at 37 ºC. The cells were seeded in 6 well plates and allowed to adhere for an hour. After the HepG2 cells attached to the plate, the cells were exposed to the 5 mT field for 48 hours. After stimulation, the cells were separated with Trypsin, diluted and mixed with an equal volume of Trypan blue stain. The number of viable cells was counted using a hemocytometer and Trypan blue assay.

The cell counting and morphology check was performed using a 100x-magnification microscope (MOTIC, AE31). The numbers of cells from the 6-well plates were statistically analyzed using SPSS software. To ensure the statistical significance and reliability of the data, we compared the *p-values* for the number of cells between the non-stimulated cell group and the AMF stimulus cell group using the Mann-Whitney U test, as shown in Table 1.

The MTT assay is a colorimetric assay for assessing cell metabolic activity. It is a cell viability assay and is mainly based on the enzymatic conversion of MTT in mitochondria [9]. The HepG2 cells were cultured in a 96-well plate exposed to the AMF stimulator in order to find the cell viability by MTT assay. The cell density was varied from 5×10^3 to 1×10^4 cells per well in the culture plate. The MTT assay was taken on plates that had six different cell densities. The optical density was found using 595 nm wavelength light.

3. Results and Discussion

Human liver HepG2 cancer cells were cultured over a

Table 1. Statistical analyses of the number of cells, proliferation rates, and p-values at the frequency of 0.5, 1, 1.4, and 2 kHz with exposure to 5 mT AMF during 48 hours. All contol number of cells was an average values obtained from 6-wells of plates, while the stimulated number of cells was the average value calculated from 12-wells of two plates. The p-values of Mann-Whitney U tests were obtained using SPSS software.

kHz	Number of cells		Proliferation Rates (%)		Mann-whitney U test
	Non-stimulated cells	Stimulated cells			
0.5	286,111 ± 22 %	270,648 ± 10 %	94.60	97.92	0.898
	390,000 ± 10 %	390,833 ± 11 %	100.21		
	414,630 ± 9 %	410,278 ± 11 %	98.95		
1	290,000 ± 10 %	271,296 ± 7 %	93.55	93.39	0.128
	265,185 ± 7 %	247,222 ± 11 %	93.23		
1.4	320,926 ± 6 %	261,296 ± 10 %	81.42	68.77	0.000
	257,222 ± 17 %	204,630 ± 7 %	79.55		
	210,556 ± 18 %	126,481 ± 18 %	60.07		
	249,444 ± 7 %	162,963 ± 8 %	65.33		
	333,333 ± 17 %	209,630 ± 12 %	62.89		
2	229,074 ± 9 %	215,556 ± 8 %	94.10	95.00	0.265
	275,185 ± 13 %	263,889 ± 12 %	95.90		

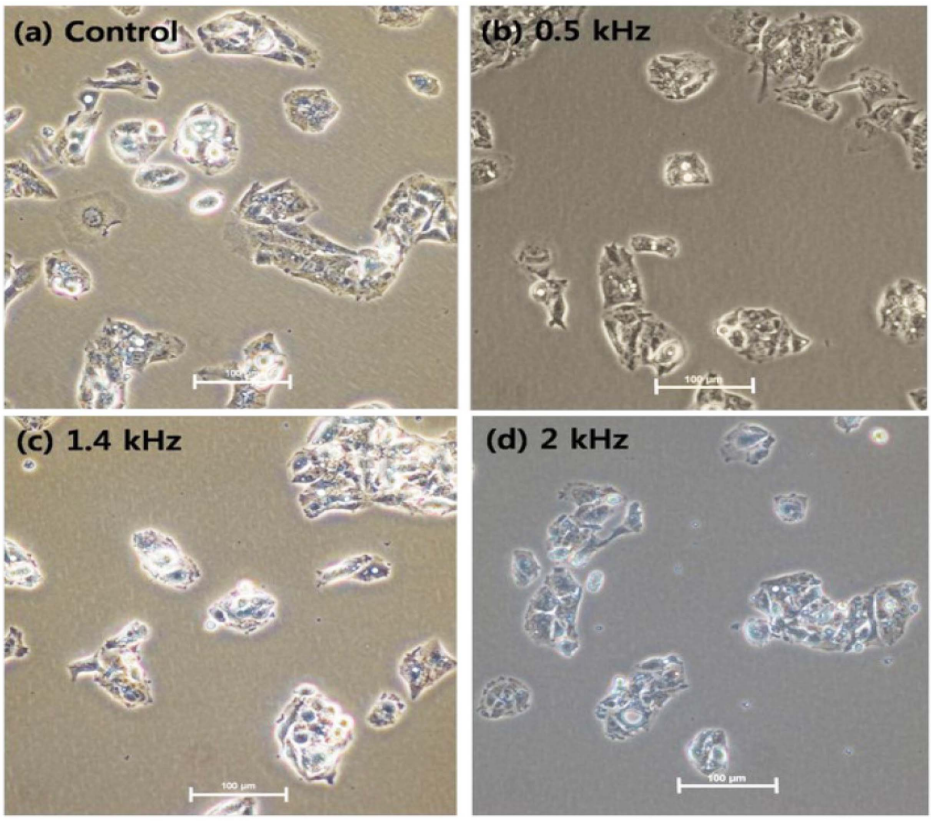

Fig. 3. The morphologies of the HepG2 cells for (a) the unstimulated control cells and the cells exposed to AMF stimulation with frequencies of (b) 0.5 kHz, (c) 1.4 kHz, and (d) 2 kHz.

magnetic coil generating an alternating magnetic field with a frequency in the range of 0.5 to 2 kHz in order to elucidate the cells proliferation rate's dependence on the frequency of the magnetic field. Figure 3 shows the morphologies of the (a) non-stimulated cells and the cells exposed to a magnetic field with an intensity of 5 mT at frequencies of (b) 0.5 kHz, (c) 1.4 kHz, and (d) 2 kHz for 48 hours. We were unable to find significant changes of morphology in any of the cell groups. In contrast, Luciana Dini *et al.* mentioned morphological modification, *i.e.* changes in cell shape, cytoskeleton, and apoptosis, of the HepG2 cells exposed to a static magnetic field of 6 mT [5]. They reported that HepG2 cells that were not exposed had a flat, polyhydric shape, were tightly attached to the culture plate, and had tiny short micro villi that were randomly distributed on the cell surface. After 24 hours of exposure to 6 mT, the exposed cells lost their polyhydric shape and acquired a fibroblast-like shape. The cytoplasm concentrated around the nucleus making this part of the cells thicker and rounder. In summary, they mentioned that the cell shape was extensively modified over the 24 hours of exposure to the 6 mT static magnetic field.

In our work, the cell shape did not change dramatically over the 48 hours of exposure to 5 mT AMF at any of the frequencies tested. The non-exposed cells remained of polyhydric shape and tightly attached to the culture plate. The difference between our results and those of Dini's research groups is only the waveform type between their static and our alternating magnetic field. This proves that the waveform of the magnetic field used is very important in cell culture.

Table 1 shows a statistical analysis of the number of live cells, the data for this analysis was collected using hemocytometer and trypan blue stain. The number of non-stimulation and stimulation cell groups were measured after exposure to fields with frequencies of 0.5, 1, 1.4, and 2 kHz. All non-stimulation cell group values are the average number of cells obtained from the 6-well plate, while the values for the stimulation groups come from the average calculated from 12 wells over two plates. As mentioned previously, if the difference in the number of cells between the two plates is over 15 %, we withdrew those results from the statistical data. The final number of cells after stimulation at 0.5 and 1 kHz showed virtually no change in comparison with the non-stimu-

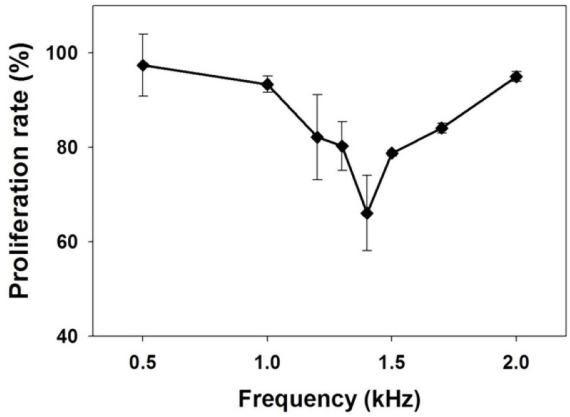

Fig. 4. The proliferation rates of HepG2 cells exposed to 5 mT AMF stimulation with frequencies ranging from 0.5 to 2 kHz after 48 hours of exposure. The proliferation rates of the stimulated cells were suppressed by up to 68 % at 1.4 kHz.

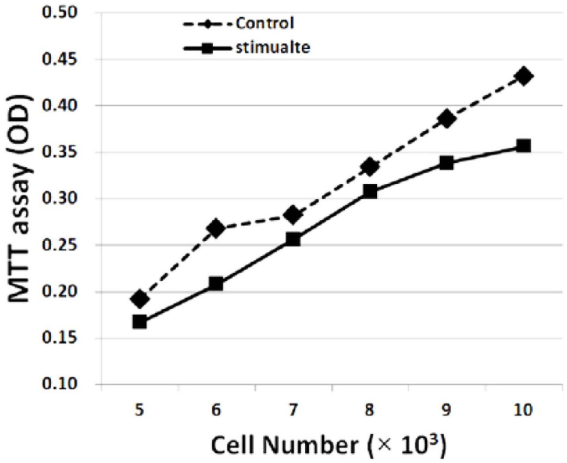

Fig. 5. The MTT assay; OD (optical density) of the HepG2 cells stimulated at 1.4 kHz, where the optical wavelength used was 595 nm.

lated cells, reaching 97 and 93 % of the number of non-stimulated cells, respectively. However, the number of cells in the group that was stimulated at 1.4 kHz had decreased down to 68.7 % of the number non-stimulated group cells. To get reliable results for the 1.4 kHz tests, we repeated this cell culture experiment five times over a period of two months.

The results are statistically significant when the p-value is less than the significance level, which is chosen before data collection and is usually set to 0.05 (5 %) [10]. The p-values at 1.4 kHz were 0.000. This means that we can say the proliferation of the HepG2 cells was significantly suppressed by AMF stimulation of 1.4 kHz, even if the reason for this cannot yet be explained clearly.

Figure 4 shows the frequency dependence of AMF stimulation on the proliferation rates of HepG2 cells from 500 Hz to 2 kHz. The remarkable point in this figure is the resonance phenomenon we see at 1.4 kHz. At 2 kHz, the proliferation ratio had recovered up to 95 % similar to how it was at 500 Hz. To confirm this resonance phenomenon at 1.4 kHz, the proliferation ratios of HepG2 cells were additionally measured at 1.2, 1.3, 1.5, and 1.7 kHz, as shown in Fig. 4. Even if these results have some error, it is clear that the proliferation of HepG2 cells was suppressed by AMF stimulation at the specific frequency of 1.4 kHz. We don't know the reason for this yet. However, we can deduce that it is related to ion flow in the channel of the cellular membrane, and also related to how the alternating magnetic field affects the dynamics of ions in those membrane channel. In general, the relaxation time of K^+, Na^+, and Ca^{++} in and out of the cellular membrane is almost 10^{-3} sec. Therefore, the resonance phenomenon at 1.4 kHz seems to be related with the refractory period of these ions. The refractory period is the period of time during which a cell is incapable of repeating a particular action. It is the recovery time an excitable membrane needs to be ready for a second stimulus once it returns to its resting state. Recently, numerous experimental investigations have looked into the interaction between Ca^{++} fluxes and magnetic field. The magnetic field could increase capacitive Ca^{++} influx in cells. It had been reported that the cytosolic Ca^{++} concentration of human glioblastoma cells increased when a 6 mT magnetic field was applied to the cells [11]. We need to study the dynamics of ion flow in cellular membranes in future work.

We compared the absorbance (optical density) between the non-stimulated HepG2 cells and the cells stimulated at 1.4 kHz as a function of cell density from 5×10^3 to 1×10^6 cells per well, as shown in Fig. 5. MTT assay is the most common experimental method to detect cell viability after exposure to toxic substances. The intensity of optical density in the MTT assay is closely related to the number of healthy cells. Figure 5 shows statistically significant differences in cell viability between the stimulated cells and the non-stimulated ones. The number of livings cells was clearly reduced by AMF stimulation at 1.4 kHz. The viability of the cells at 1.4 kHz was decreased as compared with the non-stimulated cells. Therefore, we can assert that AMF stimulation suppresses the proliferation of cancer cells at a specific frequency. In this work, the proliferation of hepatocellular carcinoma cells (HepG2) was suppressed by up to 68 % using AMF stimulation at 5 mT and 1.4 kHz.

4. Conclusion

The effects of AMF stimulation on the proliferation of HepG2 liver cancer cells at various frequencies from 500 Hz to 2 kHz were investigated in an incubator under a magnetic field strength of 5 mT. We found that HepG2 cancer cell proliferation decreased to 68 % of the control after application of AMF stimulation over 48 hours at 1.4 kHz. The phenomenon of suppressed cell proliferation at a certain frequency has never previously been reported. We assume that this effect is related to the dynamics of various ionic flows in the cell membranes. In order to understand this phenomenon clearly, we need further studies using Western blot into the detection of specific proteins suppressing cell proliferation and into intercellular Ca^{+2} levels.

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