Combined Effects of Gamma-irradiation and Hyperthermia on the Human Cell Lines for Various Temperatures and Time Sequences

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We tried to establish the theoretical basis of clinical use of combined modality of hyperthermia and radiation therapy. For this purpose, we made an in vitro experiment in order to get the synergistic and/or additive effects on the cell killing of hyperthermia combined with radiation therapy by using the microwave-hyperthermia machine already installed at our department. In our experiment, we use two human cell lines: MKN-45 (adenocarcinoma of stomach) and K-562 (leukemia cell lines). In cases of combined treatments of hyperthermia and gamma-irradiation, the therapeutic effect was the highest in the simultaneous trial. Hyperthermia after gamma irradiation showed slightly higher therapeutic effect than that before irradiation without significant difference, but its effect was the same in the interval of 6 hours between hyperthermia and irradiation. The higher temperature and the longer treatment time were applied, the higher therapeutic effects were observed. We could observe the thermoresistance by time elapse at 43°C. When hyperthermia was done for 30 minutes at the same temperature, thermal enhancement ratio (TER) at D0. 01 (dose required surviving fraction of 0.01) were 2.5 ± 0.08, 3.75 ± 0.18, and 5.0 ± 0.15 at 43°C, 44 °C, and 45°C respectively in K-562 leukemia cell lines. Our experimental data showed that more cell killing effect can be obtained in the leukemia cell lines, although they usually are known to be radiosensitive, when treated with combined hyperthermia and radiation therapy. Furthermore, our data show that leukemia cell lines may have various intrinsic radiosensitivity, especially in vitro experiments. The magnitude of cell killing effect, however, will be less than that of MKN-45.

Key Words: Hyperthermia, Gamma-irradiation, Thermal enhancement ratio, MKN-45, K-562 cell lines, Cell survival curve in vitro

INTRODUCTION

Over the past 10 years there has been a growing interest in the use of hyperthermia as a new modality for cancer therapy. Hyperthermia can be introduced in conjunction with conventional radiation therapy to patients who have already failed previous radiation therapy or who have advanced tumors that could not be extirpated by conventional radiation therapy, since the facts that hyperthermia might overcome the limitations of the low-LET radiation by its own biological advantages have been illuminated.

It has been well known that hyperthermia gives much more damages on the cancerous tissues than normal tissues, but its mechanisms have not been fully understood. The currently accepted proposals of mechanisms are as followed[1,2].: (1) Hyperthermia has more cytotoxic effect on the hypoxic cells with low pH than well-oxygenated cells, (2) "S" phase cells which show the resistance to irradiation are much more sensitive to hyperthermic treatment, (3) Selective increase in temperature by decrease in blood flow within tumor tissues in comparison with the surrounding normal tissues, (4) Hyperthermic treatment after irradiation inhibits the repairs from both sublethal damage and potentially lethal damage produced by radiation.

Many clinical results acquired with hyperthermic treatment alone have been accumulated over the past 10 years. Most clinical experience has been limited to the local treatment of superficial lesions. When hyperthermia alone was applied, tumor control rate of about 50% was reported[3,4] (complete response was 10%). These low control rates, even so, were obtained only in cases of application of high temperature and simultaneously severe heat

injury was observed in the surrounding normal tissues. These facts showed the limitation of the sole application of hypertherrmia. Therefore, the role of hyperthermia, at present, is being highlighted as a kind of sensitizer which can increase the local effects of radiation therapy when combined with irradiation.

Our experiment was designed to establish the basic principle for clinical application of hyperthermia combined with radiation therapy by observing the change of cell survival curves and the additive effect of hyperthermia when combined with gamma-irradiation. We used two human cell lines one of which was adenocarcinoma of stomach and the other of which was human leukemia cell line. Stomach cancer, which has the highest incidence in Korea, has a tendency to spread frequently to adjacent tissues and sometimes is difficult to be extirpated completely by surgery. In this case, radiation therapy is indicated, but it also has some limitations because of low tolerable dose of surrounding normal tissues (TD5/5:4,500 cGy/5 wks). Hyperthermia, therefore, is required combined with radiation therapy.

MATERIALS AND METHODS

1. Cell Lines

MKN-45 cell, poorly differentiated adenocarcinoma of stomach, and K-562, chronic myelocytic leukemia cells, were used in our experiment. MKN-45 cells were donated from Dr. Saji (National Cancer Institute of Japan) and cultured continuously at the laboratory of Immunology in our hospital. K-562 cell line were purchased from ATCC (American Type Culture Collection, CCL 243). Two cell lines were grown in RPMI-1640 media (Gibco lab., USA) supplemented with 10% fetal calf serum (Gibco lab.) (in case of K-562, 20% FCS), penicillin-streptomycin (1×10^2 U/ml, Gibco lab., USA), NaHCO (Merk Co., Germany) 24 mM/l, and HEPES buffer solution (Sigma Co., USA). This culture was incubated at 37°C in 5% CO_2 in air and subcultured by trypsinization twice weekly.

2. Preparation of Cells

We used the exponential-phased cells in our experiment. These cells were inoculated in the media at 5×10^4 viable cells/5 ml media in each T-25 flask (Costar Co., USA) without addition of new media (Unfed culture). Cultured cells were grown and we plotted the number of grown cells on the semilogarithmic scale (cultured time vs total number of viable cells in the flask). Finally, we used cells in the mid-exponential phase which corresponded to 3 days after commencement of culture.

3. Measurement of Cell Number

Cell numbers were measured with trypan blue exclusion method.

1) MKN-45 cell

After we removed the media in the flask and washed the bottom-lined cells 2-3 times with sterile phosphate-buffered saline (PBS; pH 7.2), we added 0.25 ml mixture of 0.05% trypsin (1:250)-0.53 mM EDTA (Gibco lab., USA), left for 5-10 minutes in the incubator and separated the cells from bottom of flask. Afterthen, 5 ml of new media was put into T-25 flask to stop the action of trypsin and cells were allowed to be separated each other by pipetting 5-6 times. After 50 ul of single cell suspension was mixed into the same volume of 0.4% trypan blue (Gibco lab., USA) and micro-pipetted 5-6 times, it was put on two chambers of hemocytometer (Improved Neubauer, American Optical Co., USA) and counted with microscope. We counted blue-stained cells as non-viable ones and non-stained cells as viable ones.

2) K-562 cell

We got the cells directly from T-25 flask because they are usually growing as a single cell suspension and mixed them with trypan blue and counted viable cell number.

4. Irradiation

Gamma-irradiation was done at room temperature with Co-60 teletherapy unit (AECL 780, Canada). Cells were irradiated at 0.5 cm depth in 30×30 cm of field size and the dose rate was 100 cGy/min. In order to get the backscattered dose enough, we put T-25 flask on the tissue-equivalent material of 10 cm in thickness. We also tried to let the maximum absorbed dose located on the surface of medium by putting the bolus of 0.5 cm in thickness on it. Various doses of single exposure were given to cells. In case of MKN-45, 1, 2, 3, 4, 5, 7, 9 and 10 Gy were given and, in case of K-562, 3, 6, 9, 12 and 15 Gy were given respectively.

5. Hyperthermic Treatment

Hyperthermic treatment to cells was done in the water bath of 18 l in volume (Techne B-18; Techne Co., UK). Centrifuge tube (15 ml; Corning Co., USA) containing single cell suspension was allowed to locate at 5 cm depth beneath water surface in the state of fixation with wire. For maintaining constant

temperature within the range of 0.01°C, we connected the water bath to digital immersion circulator of 10 l/min in circulation power (Tempette TE-8D, Techne Co., UK). In this situation, we measured the temperature of media in the centrifuge tube with digital thermometer (BAT-8, Baily Co., USA). It took, on the average, 5 minutes to rise up to 43°C and so we considered the total heating time as time spanning from immersion of centrifuge tube into water bath plus 5 minutes. Digital immersion circulator and digital thermometer were used after calibration with standard temperature calibrator (Promac DHT-830S, Canada) and stability of temperature was, at the same time, confirmed with a calibrated mercury-thermometer of 0.1 unit (Anschutz, Germany) at each experiment. Cells were cultured without addition of new media or exchange of media during period between irradiation and hyperthermic treatment.

6. Measurement of Cell Survival Rate

Survival rate of cells was calculated with Spearman-Karber method. We inoculated 3×10^4 cells into 100 ml media in each 8 wells of first column of 96-well microplate, followed by dilution to 1/3 of cell density in the just preceding column until reaching the 12th column. After this constant dilution of cells, cells in the 96-well microplate were cultured for 14 days in the incubator and then observed with phase-contrast microscope (Biostar, American Optical Co., USA). At this time, we considered colonies composed of more than 50 cells as the evidence of proliferation of viable cells

after irradiation and hyperthermic treatment. On the basis of it, we calculated the number of colony formation in each wells.

The equation for surviving fraction (SF) by Spearman-Karber method is as followed:

$$\Phi = e^{-0.577 - (Xo + d/2 - d \times Pi)}$$

Xo = ln Z (Z: No. of cells/1st column of each well)
d = ln a (a: dilution coefficient = 3)
Pi = ri/ni (= positive rate/heat)
ri = No. of wells in which colony was formed
ni = No. of wells/column (= 8)
SF = Φ irradiation and/or heat/Φ control

RESULTS

All of our data points were obtained after experiments were repeated 5 times independently and represented mean values ± 5% S.D..

1. Survival Rate of Cells by Irradiation

The survival rate of MKN-45 cells was 0.3 at 2 Gy (SF2 = 0.3) and that of K-562 was 0.12 at 2 Gy (SF2 = 0.12). This finding showed that K-562 has a higher radiosensitivity than MKN-45 (Fig. 1, 2) ($p < 0.05$)

2. Survival Rate of Cells by Hyperthermia

The survival rate of K-562 leukemic cells was, according to treatment temperature and treatment time, within the range of 0.07 at 43°C when heated for more than 90 minutes and showed thermotoler-
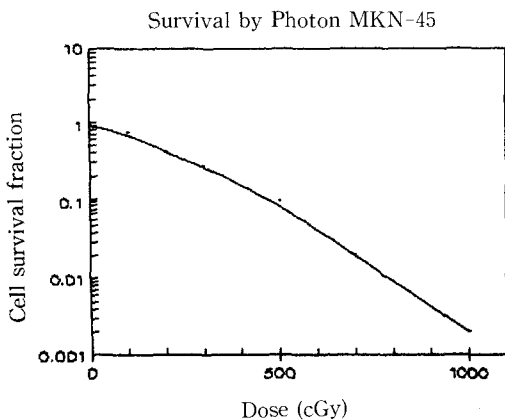

Fig. 1. Cell survival curve of MKN-45 adenocarcinoma cell line by gamma irradiation. This experiment was repeated independently 5 times and each points represents mean value ± 5% SD.
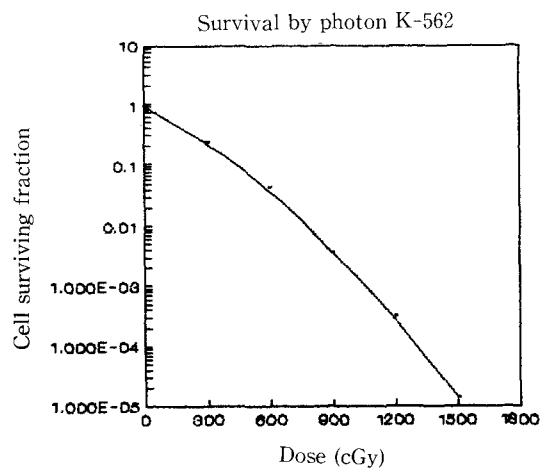

Fig. 2. Cell survival curve of K-562 leukemia cell line by gamma irradiation. This experiment was also repeated independently 5 times and each points represents mean value ± 5% SD.

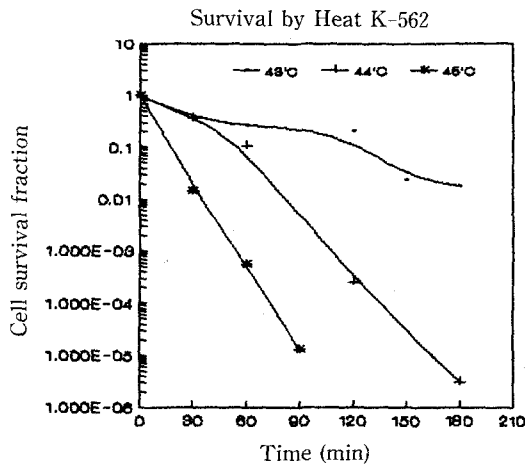Survival by Heat K-562

Fig. 3. Cell survival curve of K-562 leukemia cell line by hyperthermia alone. Each point represents mean value±5% SD.

Combination of Heat and Photon MKN-45

Heat-Radiation-Heat

Fig. 4. Cell survival curve of MKN-45 adenocarcinoma cell line by thermoradiotherapy with gamma irradiation according to time interval between radiation and hyperthermia

ance to hyperthermic treatment. At temperatures of both 44°C and 45°C the survial rate was 0.00001, which is a measurable limitation by Spearman-Kerber method, when heated for 90 and 150 minutes (Fig. 3).

3. Change of Survival Rate by Combined Treatment of Irradiation and Hyperthermia

We obseved the change of survival rate of MKN-45 by virtue of time intervals between irradiation and hyperthermia when treated with combined
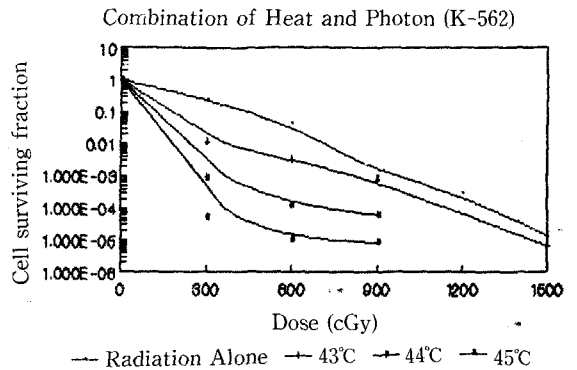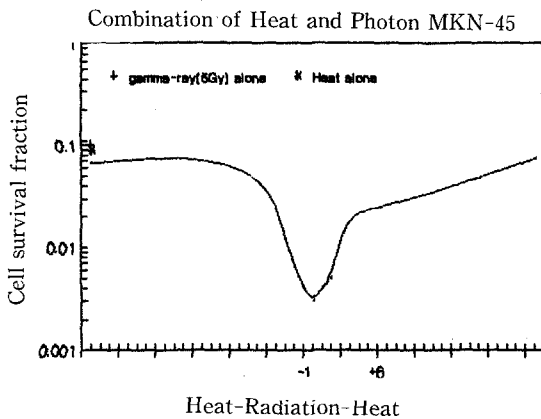
Combination of Heat and Photon (K-562)

Fig. 5. Cell survival curve of K-562 leukemia cell line by thermoradiotherapy. Each point also represents mean value±5% SD.

therapy. The survival rates for single exposure of 5 Gy gama-irradiation and hyperthermia at 43°C for 30 minutes were 0.1 and 0.089 respectively. When treated with combined therapy, its survival rate was 0.069 and was the lowest as 0.003 in case of no time interval between irradiation and hyperthermia (simultaneous treatment). This fact showed the additive effect of hyperthermia on effect of irradiation (additive cell killing effect). When time sequence was considered, hypertherrmic treatment before radition showed better cell killing effect and had a statistical significance within 3 hours of time interval (Fig. 4).

In case of K-562 leukemic cells, combined therapy with irradiation and hyperthermia showed better cell killing effect than radiation alone. At this time hyperthermia was done for 30 minutes at 43°C, 44°C, and 45°C. TER at D 0.01 were 2.5±0.08, 3. 75±0.18, and 5.0±0.15 respectively, and 1.22±0. 06, 3.30±0.16, and 5.5±0.17 at D0.001 (Fig. 5).

As a result of the above-mentioned findings, we concluded that hyperthermia combined with radiation therapy could increase the therapeutic effect and a limited control rate of MKN-45 (adenocarcinoma of stomach) would, although it usually shows the resistance to irradiation alone, be overcome with combined therapy. In addition, we observed the thermotolerance and that TER had a proportional correlation with both treatment temperature and treatment time until reaching thermotolerance.

DISCUSSION

Broad experience has been rapidly accumulated in the clinical use of localized hyperthermia,

either with external or interstitial delivery techniques. But most of the clinical experience over the past 10 years has been limited to the local treatment of the superficial lesions because of the dearth of equipment to adequately deliver heat to the deep-seated tumors, the technical difficulties in measuring temperatures in large volumes, and some patient intolerance. This is related to the inherent limitations of the existing equipment, which allows for satisfactory power deposition at depths about 3 to 4 cm of the body's surface or in sites that are accessible for interstitial implantation. Therefore, it is impossible to cure the patients with deep-seated tumors or large volume of tumors completely with hyperthermic treatment only. With hyperthermia alone, complete responses in the range of 15% have been described, usually of short duration[3,4]. Furthermore, this complete response rate was limited to small superficial tumors. In cases of Marmor et al[5], the complete response rate was 11% (5/44 patients) with ultrasound heating.

Accordingly, hyperthermic treatment combined with irradiation was highlighted for the past 10 years. Most of clinical articles reported that the complete response has ranged from 47% to 87% (average: 70%) with irradiation and hyperthermia, showing about 2-fold increase in complete response rate with irradiation alone[4].

Several experiments combining hyperthermia and ionizing radiation in vitro have demonstrated that the interaction of these modalities depends on a large number of factors; temperature, time at the elevated temperature, sequence of heat and radiation treatment, and time interval between these treatments, and/or the dose rate and quality of radiation[6]. Robinson's report[7], one of the initial studies of the effects of various elevated temperatures and treatment sequences on radiation sensitivity, showed that sensitization was significant at 40°C and maximum radiation sensitizing effect might occur with increasing heating time. Our experiment also showed the same results as Robinson's one. Most of experiments, although some exceptions have been reported, demonstrated that the sensitizing efficacy of hyperthermia increased with temperature if the time of treatment at elevated temperature was constant[7~10]. Also in our experiment, thermal enhancement ratio (TER) at 30 minutes treatment time were 2.5, 3.75, and 5.0 at 43 °C, 44°C, and 45°C respectively, when surviving fraction was 0.01. These values of TER were higher than ones reported till now by other researchers

(reported maximum enhancement ratios were approximately 2)[6~11]. We think that its cause appears to be the use of different cell lines--in our experiment, K-562 (leukemia cell lines) was used. If we should have calculated the TER of MKN-45 (adenocarcinoma of stomach), its TER value would have been much lower than K-562.

Studies by Robinson and Wizenberg[7] have shown that equivalent thermal sensitization to radiation cell killing if hypertermia was done before or after irradiation. In contrast, we observed in both MKN-45 and K-562 cell lines that hyperthermia was more effective when delivered simultaneously with irradiation or after irradiation than before irradiation. Our results were dissimilar to Dewey's observation[8]--Dewey and Gerweck[8] reported that cell killing effect was higher when hyperthermia was delivered before irradiation than after irradiation. Our observation, however, had statistically no significant difference between before and after irradiation. Joshi, Barendsen, and van der Schueren[11] observed that the relative efficacy of hyperthermia before or after irradiation had varied with the treatment temperature and/or magnitude of the heat treatment, but we did not execute such experiments. In case of MKN-45 cell lines, we observed the relative efficacy of heat at 43°C for 30 minutes before or after irradiation of 5 Gy (Fig. 4). Survival following combined treatment was less than hyperthermia alone or irradiation alone, and the least when combined treatment was done simultaneously. These findings of the greater killing efficacy have also been observed in K-562 cell lines.

In these cell lines, when combined therapy was given simultaneously, the cell killing effect increased with magnitudes of both the heating temperature and radiation dose (Fig. 5). More interestingly, thermotolerance appeared in the high dose region (more than 5 Gy) when the temerature of delivered heat had been risen up to 44°C--Cell survival curves reached the plateau phase. The experiment to elucidate the exact mechanism for thermotolerance was not carried out in the present study.

Repeatedly speaking, the time sequence is very important in cell killing. As seen in figure 4, we observed very much sensitization or interaction between heat and radiation when three hours separated the treatments. In studies performed at higher temperature by Gerweck et al[6], where the lethal effects of heat alone were more pronounced,

hyperthermic treatment sensitized cells to radiation for up to 15 hours after treatment. However, we could not observe these findings in our experiment and rather survival increased relatively quickly when heat was delivered more than 3 hours before or after irradiation. Consequently, it is reasonable that the time interval between heat and radiation should be decreased, at least, up to 3 hours in order to increase the killing efficiency of the combined modalities. The lethal interaction of heat and radiation was most pronounced when cells were treated with both agents simultaneously and the thermal sensitizing efficacy increased with increasing temperature if the heating time was constant, and the synergistic interaction between heat and radiation appeared to decay when more than 3 hours separated the treatments.

When hyperthermia was combined with low-LET radiation, the killing effects of radiation was enhanced. The main cause of this phenomenon may be that repair of sublethal radiation damage can be blocked by sufficient hyperthermic treatment. This fact suggest that the radiation enhancement effects of hyperthermia would be most pronounced under radiation exposure conditions where repair of sublethal radiation damage is prominent, as occurs with low-LET radiation[6]. Similarly, the accumulation and repair of sublethal damage is less prominent in cells exposed to high-LET radiation and, therefore, the radiosensitizing effects of heat would be expected to be less prominent with radiation of this quality[12,13]. To testify the above-mentioned findings, we have done another experiment with 50 MeV neutron generated from cyclotron which had been already installed in our department. Our results showed, as suggested, that the magnitude of radiosensitizing effect was less prominent than that of low-LET radiation (gamma-ray) when combined with hyperthermia. The report on more detailed findings and explanations will be published in the near future.

CONCLUSION

We have studied the radiosensitizing effect of hyperthermia combined with irradiation on MKN-45 cell lines (adenocarcinoma of stomach) and K-562 cell lines (leukemia). For making this effect clear, we have emphasized the time interval between hyperthermia and irradiation, the time sequence, and the magnitude of radiosensitizing effect of heat (represented by thermal enhancement ratio: TER).

We also compared the cell killing effect of combined treatment to those of hyperthermia alone irradiation alone.

1) The cell killing effect of combined treatment was superior to that of hyperthermia alone or irradiation alone in both cell lines (MKN-45 and K-562 cells): In case of MKN-45 cells, the survival rates by 5 Gy gamma-irradiation alone and hyperthermia alone at 43°C for 30 minutes were 0.1 and 0.089 respectively, whereas the combined treatment showed 0.069 survival rate (this value represents the maximum).

2) The cell killing effect of hyperthermia increased as the temperature of heating and heating time increased, but thermotolerance appeared when heat was delivered at more than 43°C.

3) The time interval between heat and irrdiation was a very important factor determining the radiosensitizing effect of hyperthermia: when heat was delivered simultaneously with irradiation, the synergistic effect of combined treatment was the highest. Within 3 hours, the effect of combined treatment showed a statistically significant change.

4) In MKN-45 cell lines, hyperthermic treatment following irradiation had higher cell-killing effect than hyperthermia before irradiation without statistically significant difference, especially when the time intervals were separated more than 6 hours.

5) In K-562 cell lines, to which heats were delivered for 30 minutes at 43°C, 44°C, and 45°C, TERs (thermal enhancement ratios) were 2.5 ± 0.08, 3.75 ± 0.18, and 5.0 ± 0.15 at D 0.01 respectively and 1.22 ± 0.06, 3.30 ± 0.16, and 5.50 ± 0.17 at D 0.001 respectively.

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= 국문초록 =

감마선과 온열치료 병용시 세포 치사 능력 증강에 관한 실험적 연구

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원자력 병원 치료방사선과에서 사용 중인 극초단파 온열치료장치에 의한 온열치료시 온열치료의 적정화로 암 치료기술 향상을 도모함을 최종 목표로 하여 인체 암세포주에서 방사선과 온열에 의한 세포생존곡선의 기본적인 특징과 방사선과의 병용에 따른 치료효과의 증강을 파악하여 임상적 응용의 기초적인 근거를 확립하고자 하였다. 본 실험에서는 2개의 세포주, 즉 위 선암 세포주와 만성 골수성 백혈병 세포주를 사용하였다. 위암은 한국인에서 가장 발생빈도가 높고, 국소적으로 진행된 경우, 주위 장기의 한계선량으로 인하여 방사선 단독으로는 적용의 한계가 있어 온열치료와의 병용이 요구되며, 백혈병 세포는 방사선에 민감한 대표적인 세포의 하나로 온열치료와의 병용시 발생할 수 있는 미묘한 차이를 보다 분명히 관찰할 수 있을 것으로 기대하여 본 실험에 사용하였다.

감마선과 온열치료를 겸한 경우 두가지 방법을 동시에 시행한 경우, 치료효과가 가장 높았고 온열을 감마선 치료 후에 시행한 경우가 감마선 치료 전에 시행한 경우보다 약간 높았으나, 그 차이는 크지 않았고, 6시간 이상의 간격에서는 같았다. 온열치료의 온도 및 치료시간과 치료효과는 온도가 높을수록 치료시간이 길수록 치료효과가 높았고, 43°C에서는 온열내성을 시간경과에 따라 볼 수 있었다.

같은 온도로 30분간 온열치료시 온열치료효과 증진비는 섭씨 43도, 44도, 45도의 경우, D 0.01을 기준으로 2.5 ± 0.08, 3.75 ± 0.18, 5.0 ± 0.15였다.

본 연구는 기초 생물학적 실험이나, 이 결과는 임상 암치료에 직접 이용되는 것이며 또한 치료 수행에 반드시 필요한 과정이다. 따라서, 치료방법의 결정, 성적분석, 치료 부작용의 예측 및 대책 확립 등에 적용되고 본원 온열치료의 특성 제시에 활용될 수 있다.

주요단어 : 온열, 감마선, 온열효과증가비, MKN-45, K-562, 세포생존곡선