Enhancement of *in vivo* Radiosensitization by Combination with Pentoxifylline and Nicotinamide*

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Pentoxifylline (PENTO) has been known to improve RBC fluidity, and thus improve the flux of RBC through narrow capillaries. Additionally, PENTO also decreases the O_2 affinity of hemoglobin by increasing 2,3-DPG levels, thereby increasing the O_2 release from RBC. Nicotinamide (NA) has been reported to decrease the number of acutely hypoxic cells in tumors by temporarily increasing tumor blood flow. Therefore, the purpose of this study was to examine whether the combination of PENTO and NA (PENTO+NA) would reduce the radioresistance of the FSaII murine fibrosarcoma by oxygenating the hypoxic cells. We observed a significantly enhanced radiation-induced growth delay of the FSaII tumors by PENTO+NA. Thus the enhancement ratio was between 2.5 and 2.8 in growth delay assay. The TCD_{50} of control tumors was about 57 Gy, but that of PENTO+NA treated tumors was about 32 Gy. Thus TCD_{50} was modified by a factor of 1.8. We also observed that PENTO+NA exerted no effect on the radiation-induced skin damage after the legs without bearing tumors were exposed to X-irradiation. In order to clarify radiosensitizing effects of PENTO+NA, changes in tumor blood flow and intratumor pO_2 were measured using laser Doppler flowmetry and O_2 microelectrode methods. The tumor blood flow significantly increased at 10 min. after injection of PENTO+NA. Furthermore, we also found that PENTO+NA significantly increased intratumor pO_2 from 8 to 19 mmHg. We concluded that PENTO+NA was far more effective than NA alone or PENTO alone. The increase in the response of tumors ***in vivo*** to X-irradiation appeared to be due mainly to an increase in the tumor oxygenation. Further studies using various concentrations of PENTO alone and in combination with NA to obtain better sequencing and maximal radiosensitization are warranted.

Key Words: Radiosensitization, Tumor oxygenation, Pentoxifylline, Nicotinamide, Intratumor pO_2, Laser Doppler flowmetry, RBC flux.

INTRODUCTION

Chronically and acutely hypoxic cells in solid tumors are generally considered to be a major problem in the treatment of cancer by radiation therapy[1~3]. Pentoxifylline (PENTO), a derivative of methylxanthine, has been widely used in the treatment of claudication[4,5], peripheral vascular disorders[6], cerebrovascular disorders[7], sickle cell anemia disorders[8] and a number of other disorders[9]. PENTO has been reported to improve red blood cell (RBC) fluidity, and thus improve the flux of RBC through narrow capillaries[5,8~10]. Additionally, PENTO also decreases the O_2 affinity of hemoglobin by increasing 2,3-diphosphoglycerate (2,3-DPG) levels, thereby increasing the O_2 release from RBC[11]. Furthermore, PENTO has no serious toxicity in patients[9,12]. Nicotinamide (NA), also known as the amide form of nicotinic acid, has been used clinically for the treatment of several disorders including pellagra[13] and schizophrenia[14,15]. Additionally, several investigators have demonstrated that a single dose of NA (200~1000 mg/kg) can significantly enhance the radiation damage in several murine tumor systems[16~18].

We hypothesized that PENTO may increase the oxygenation of tumors by increasing the flux of RBC through narrow tumor capillaries as well as increasing the O_2 release from RBC. NA has been known to particularly oxygenate the acutely hypoxic cells by improving tumor blood flow[19]. The rationale for combining PENTO and NA (PENTO+NA) is as follows: single or multiple administrations of relatively nontoxic PENTO may primarily cause an

*This paper is dedicated to Professor Chang W. Song on the occasion of his 60th birthday.

increase in oxygen delivery to chronically hypoxic cells. The subsequent single administration of NA may result in an increase in O_2 transport to acutely hypoxic cells. Therefore, in this study we have investigated whether PENTO+NA would potentiate the radiation damage in FSaII tumors by improving tumor oxygenation. The tumor growth delay as well as tumor cure (TCD_{50}) were used as endpoints in this study. The effect of PENTO+NA on the radiation-induced damage after local irradiation in normal tissues, such as skin damage of the C3H mice was also studied. In order to clarify the radiosensitizing effects of PENTO+NA, we have studied changes in the tumor pO_2 by PENTO+NA, which were measured polarographically with O_2 microelectrodes. The tumor blood flow after treatment with PENTO+NA was also measured using the laser Doppler flowmetry.

METHODS AND MATERIALS

1. Tumors

FSaII tumors, syngeneic to C3H mice, were used. The cells in the exponential growth phase were harvested by treatment with a 0.25% trypsin solution. About 2×10^5 viable cells, suspended in a 0.05 ml medium free of serum, were injected subcutaneously into the right thigh of 8 to 10-week-old C3H female mice. Tumor ranging in size from 130 ~180 mm^3 in the host mice were used in this study.

2. Drug Treatment

Pentoxifylline (or Trental; 3,7-dimethyl-1-[5 -oxohexyl] xanthine) and nicotinamide (pyrimidine -3-carboxamide) were purchased from Sigma Chemical Co. (St. Louis, USA) and were dissolved in a sterile saline solution (0.9% NaCl) prior to each experiment. A group of mice bearing tumors in the hind legs were treated intraperitoneally (i.p.) with PENTO (100 mg/kg/day) for 1 or 3 days. The tumors were locally X-irradiated 24 hr. after the last injection of PENTO. Another group of mice were treated with an i.p. injection of NA (500 mg/kg) 1 day after the third injection of PENTO and the tumors were X-irradiated.

3. X-Irradiation

The legs with or without tumors were locally exposed to various doses of X-rays in a single exposure. The radiation factors were 250 kVp, 15 mA with an added filtration of 1.0 mm Al and 0.35 mm Cu. For local irradiation, the TSD was 50 cm, and the dose rate was 89.5 cGy/min.

4. Response of FSaII Tumors

For growth delay assay, the individual tumors were checked 2~3 times a week for 60 days with a caliper. The tumor volume was calculated with the use of the formula $V=0.4\,xAB^2$, where A and B, respectively, were the longer and shorter diameters of the tumors. When the tumor grew to an average diameter of 2 cm, the tumors were judged to be incurable and the host animals were sacrificed by cervical dislocation. Mice with no palpable tumors 120 days after treatment were judged to be cured and the TCD_{50} was calculated by logit analysis[21].

5. Response of Skin

The skin reaction in the X-irradiated legs was scored 2~3 times weekly for 40 days. An arbitrary scale to quantitate the radiation-induced skin damage as previously described was used[20]. The average scores over 40 days were calculated for each radiation dose.

6. Measurement of Intratumor PO_2 Using O_2 Microelectrodes

Polarographic measurement of tumor pO_2 was performed by using recessed tip microelectrodes with diameters of 40~50 μm. The mean sensitivity of microelectrodes was about 0.1×10^{-10} Ampere/percent O_2 at 34.0°C. These electrodes were constructed in our laboratory, as previously described[21]. Mice were anesthetized with an i.p. injection of 200 mg/kg Inactin (sodium salt ethyl-[1 -methyl-propyl]-malonyl-thio-urea). To keep the animals' body temperature around 37.5°C (tumor temperature was around 34.0°C), the mice were placed on an isothermal pad in an electrically insulated Faraday cage. In addition, a warming lamp was used to keep the body temperature of the host animals at 37.5°C.

7. Measurement of Blood Flow Using Laser Doppler Flowmetry

Blood flow was measured by the use of the laser Doppler principle. The laser Doppler device used in this study was the Laserflo® blood perfusion monitor (TSI, St. Paul, USA), which utilizes infrared light (780 ± 20 nm in wavelength) with a penetration of about 1.0~1.5 mm, and also illuminates a tissue volume of about 1.5 mm^3. Photons scattered by both moving RBCs and stationary tissue cells are collected and processed to yield electrical signals proportional to the volume and velocity of RBC and then the rate of RBC flux is determined as the

products of the volume and velocity. Using this technique, it is possible to directly and continuously observe RBC flux[22]. Although the laser Doppler flowmetry measured only RBC fluxed in superficial tissue areas, the laser Doppler method is widely used for the measurement of relative changes in the tissue blood perfusion[22~24]. The effects of PENTO+NA on the RBC flux are of special interest in this study due to the fact that the RBC flux critically influences the amount of oxygen which reaches the tumor cells. The animals bearing FSaII tumors were anesthetized with at a dosage of 200 ~250 mg/kg of Inactin. Additionally, the warming lamp was used to keep the mice body temperature at 37.5°C. For the measurements in tumors, about 5 mm in diameter of the skin over the tumor was carefully removed with a pair of fine scissors and the laser Doppler probe was placed over the exposed tumors. Electrical gel was applied to the surface of the laser Doppler probe. The probe was placed above (but not in contact with) the surface of the exposed tumors to be monitored. Therefore, tissue compression was avoided so that the microcirculation would not be disturbed[24]. After the laser Doppler signals were stabilized, 500 mg/kg of NA was i.p. injected into the saline or PENTO pretreated mice. The dynamic changes in the laser Doppler flow (LDF) signal were continuously monitored and recorded by a chart recorder.

8. Statistical Analysis

Value given in this study are means±standard errors (SE). The significance of differences within a group was evaluated with a paired t-test. The significance of the differences between different groups was tested with a unpaired t-test.

RESULTS

Fig. 1 shows changes in the mean volume of FSaII tumors as a function of days after various treatments. The time required for the tumors to reach four-fold of their initial volume was calculated. The four-fold tumor volume for untreated control tumors took 4 days. When 10 Gy was applied, the tumors increased by 4 fold in about 8 days. As a result, about a 4 day growth delay was observed by radiation (10 Gy) alone. When the mice were treated with 100 mg/kg/day of PENTO 1 day before X-irradiation of 10 Gy, the time required to reach the fourfold tumor volume was about 12 days. However, when the mice were injected daily with 100 mg/kg/day of PENTO for 3 days, the four times increase in tumor volume took about 22 days.

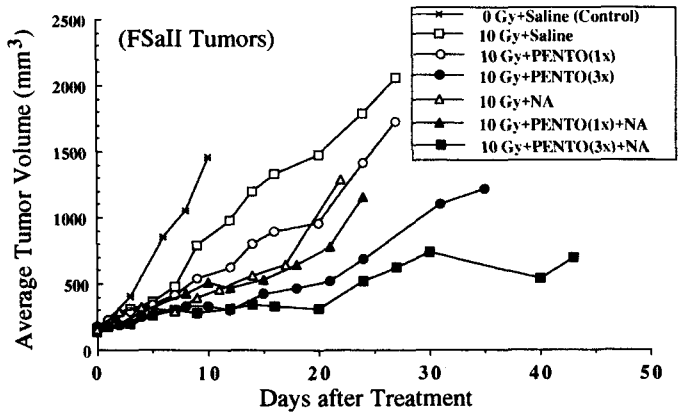

Fig. 1. Changes in the mean volume of FSaII tumors are shown as a function of days after the treatment of PENTO alone, NA alone or in combination with PENTO and NA (PENTO+NA). Open squares represent saline (10 ml/kg)+10 Gy; open triangles represent NA (500 mg/kg)+10Gy; open circles represent PENTO (1×100 mg/kg/day)+10Gy; closed circles represent PENTO (3×100 mg/kg/day)+10Gy; closed squares represent PENTO (3×100 mg/kg/day)+NA (500 mg/kg)+10 Gy. Mean of 7-14 tumors are shown; however, SE has been omitted to maintain clarity.

When mice were treated with PENTO+NA, the tumor increased by four times in volume took about 27 days.

Fig. 2 shows the effect of PENTO+NA on the tumor growth; i.e., the time taken for tumors to reach four-fold of their initial volume as a function of a range of radiation doses[20]. Increasing radiation doses resulted in an increase in the time taken for initial tumors to regrow to four times their treatment volume. For the saline (10 ml/kg) treated group, all tumors regrew up to a single exposure of 30 Gy. However, some of the tumors which were treated with PENTO+NA did not regrow by 120 days postirradiation, indicating these were cured. The rest of the tumors which were not cured grew to four times their original volume within 60 days.

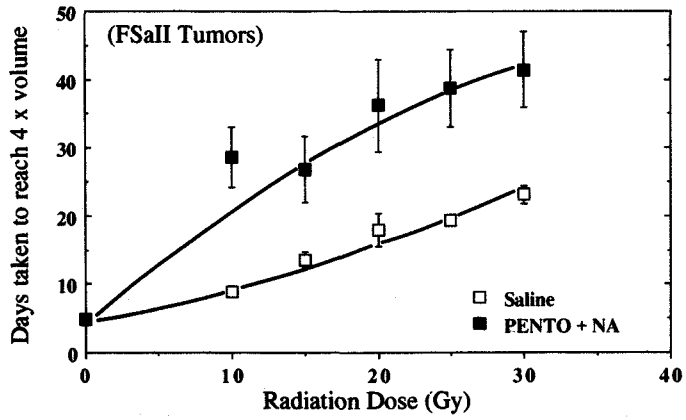

Fig. 2. Tumor growth time for the FSaII tumor in C3Hf/Sed mice before (open squares) and after (closed squares) PENTO+NA treatment are shown as a function of various radiation exposure (0-30 Gy). The tumors growth time represents the time in days required for tumors to regrow to four times their initial treated volume. Mean of 7-14 tumors and SE are shown.

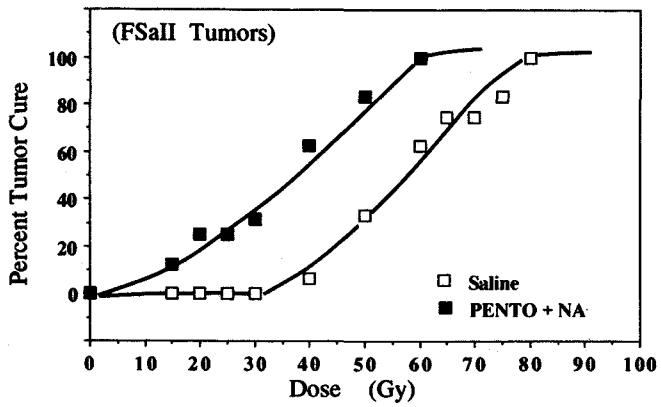

Fig. 3. The percentage local control of FSaII tumors as a function of X-irradiation dose. The animals were treated daily with injections of PENTO (100 mg/kg/day) for 3 days, and an additional injection of NA (500 mg/kg) on the fourth day, 1 hr. prior to X-irradiation (closed squares). Control animals were treated with saline (10 ml/kg), 1 hr. prior to X-irradiation (open squares).

Therefore, 60 days were taken as the regrowth analysis[20]. In fact, this was the reason the SE values for the growth delay by the higher radiation doses for PENTO+NA were rather large. Although this procedure artificially reduced the mean growth delay by the higher radiation doses, the procedure did not affect overall conclusion in this study. A large delay of tumor growth occurred when the host animals were injected with PENTO+NA, producing enhancement ratio of between 2.5 and 2.8.

Fig. 3 shows the local tumor control by X-irradiation in mice treated with saline or PENTO+NA. The percentage of FSaII tumors scored as cured in each experimental group was plotted as a function of radiation dose. The TCD_{50} in the saline treated group was 56.6 Gy with a 95% confidence interval of (55.6, 57.7) Gy. The treatment with PENTO+NA caused a greater radiosensitization as demonstrated by a reduction in TCD_{50} to 31.9 (30.8, 32.9) Gy. The dose modification factor for TCD_{50} was 1.78 for the treatment of PENTO+NA group.

Fig. 4-A shows the radiation-induced skin damage in the legs as a function of days after the treatment with PENTO+NA in a single exposure of 30 Gy. We also expanded the same protocol to various radiation doses. Fig. 4-B shows the average skin reaction in the legs during the first 40 days post-irradiation in C3H mice as a function of the radiation dose applied. PENTO+NA exerted no effect on the radiation-induced skin damage. The dose required to induce slight to moderate moist desquamation for the saline-treated control was not statistically significant ($p>0.1$) difference from that for PENTO+NA.

In order to elucidate the physiological mechanism of the potentiation of radiation damage by PENTO+NA, intratumor pO_2 were measured polarographically using microelectrode methods. Fig. 5 is a histogram of pO_2 distribution in the FSaII

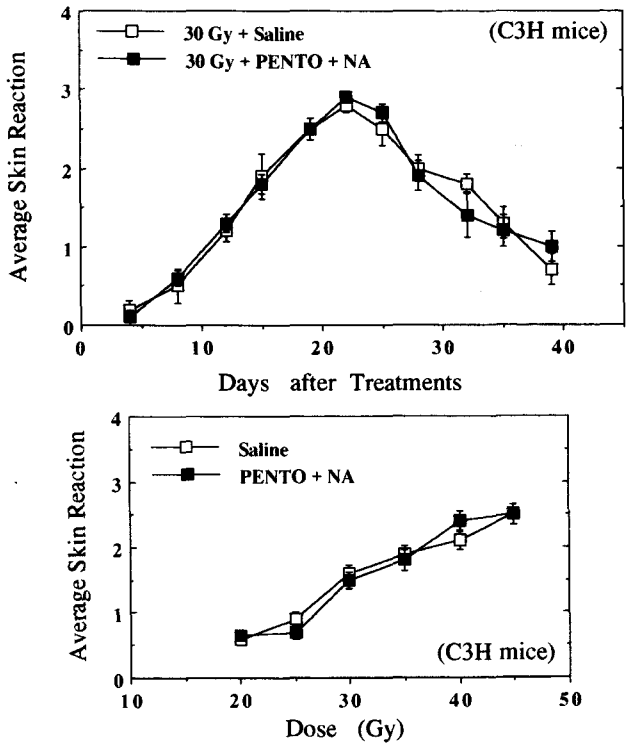

Fig. 4. The treatment of the animals and skin were same as those for Fig. 3. Mean of 7-14 non-tumor bearing mice and SE are shown.
4-A. An average skin reaction before and after treatment with PENTO+NA as a function of days in a single exposure of 30 Gy.
4-B. An average skin reaction in C3H mice during 0-40 days postirradiation as a function of X-irradiation dose.

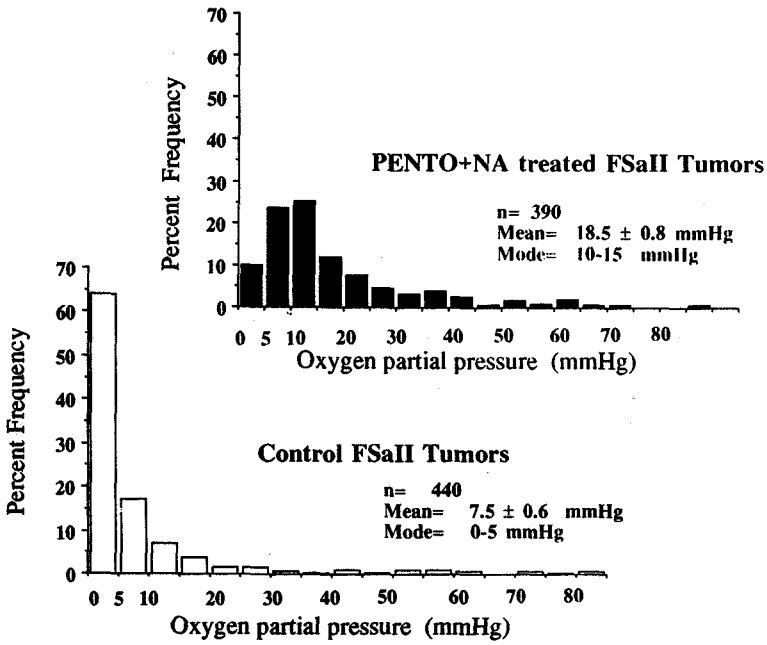

Fig. 5. Frequency distribution of measured intratumor pO_2 is constructed as a function of oxygen partial pressure (mmHg), which have grouped in 5 mmHg for each interval. A left-bottom histogram is saline (10 mg/kg) treated control FSaII tumors from 440 measurements, and a right-top is PENTO+NA treated FSaII tumors from 390 measurements.

tumors. The average pO_2 in the saline-treated control group of FSaII tumors was 7.5±0.6 mmHg (440 measurements), while that in the PENTO+NA remarkably increased to 18.5±0.8 mmHg (390 measurements). As a consequence, intratumor pO_2 in PENTO+NA was significantly different from that in the control group ($p<0.001$). It should be noted that the mode in control group was 0~5 mmHg, while that in PENTO+NA further increased to 10~15 mmHg. Moreover, the percentage of regions having low oxygen tension (i.e., less than 2.5 mmHg) significantly decreased from 28% (PENTO alone) to 1% by PENTO+NA ($p<0.001$).

As shown in Figure 6, PENTO+NA significantly increases RBC flux in FSaII tumors 10 min. after injection ($p<0.01$). In addition, NA alone significantly increased RBC flux 30 min. after the administration ($p<0.05$). PENTO alone (100 mg/kg) did not immediately increase RBC flux until 2 hr. after injection (data not shown). As a result, the percentage changes in RBC flux after the treatment with PENTO+NA appeared to be more effective than that in PENTO or NA alone.

DISCUSSION

It is a well-known fact that the tumor response to radiation therapy is closely dependent on the availability of O_2 concentration during X-irradiation[3]. When the O_2 delivery is impaired, the efficacy of radiation therapy is likely to be reduced. Treatment with PENTO alone may be involved in two physiological mechanisms to increase the radioresponse of tumors to radiation. First, PENTO alone increases the quantity of hemoglobin for O_2 transport through narrow tumor capillaries due mainly to improve RBC fluidity[5,8~10]. Second, PENTO also increases the amount of O_2 released to the tissues by reducing the hemoglobin affinity for O_2 due to the fact that PENTO alone decreases the level in 2, 3-DPG[11]. The present study demonstrated that PENTO alone (1 dose at 100 mg/kg or 3 doses at 100 mg/kg/day) can significantly enhance the effect of X-irradiation on FSaII murine tumors (Fig. 1). PENTO+NA was far more effective than PENTO

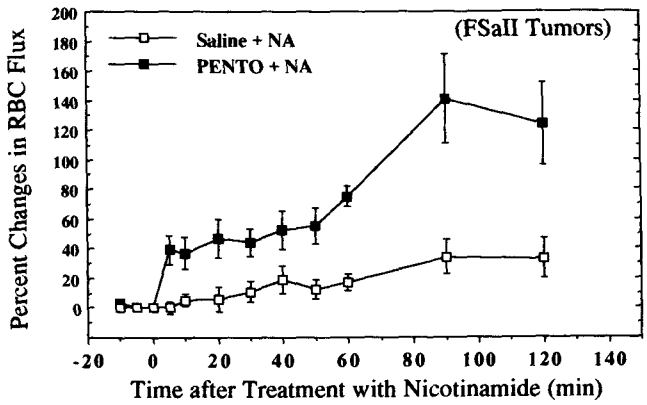

Fig. 6. Percent changes in the laser Doppler flow (RBC Flux) are shown as a function of time after various treatments. PENTO+NA (closed squares), 3 daily i.p. injections of 100 mg/kg/day of PENTO and a single i. p. injection of 500 mg/kg of NA on the fourth day. Saline+NA (open squares), i.p. injection of 10 ml/kg of saline for 3 days, and a single i. p. injection of 500 mg/kg of NA on the 4th day. Mean of 5-10 tumors and SE are shown.

or NA alone. Moreover, PENTO+NA remarkably increased the curability of FSaII tumors (Fig. 3). The TCD_{50} was significantly reduced by a dose modification factor of 1.8. However, PENTO+NA did not increase the radiation-induced damage in normal tissues such as skin damage of C3H mice for local irradiation (Fig. 4).

Recently, Fingert et al. observed that daily injections of PENTO at a dose of 257 mg/kg/day for 7 days in combination with 8 mg/kg of thiotepa enhanced the antitumor activity in human tumor xenografts without risk of increased regional or systemic side effects[25]. However, unpublished data (Lee I, 1990. 7) on the toxicity of PENTO alone showed that $LD_{50(30)}$ was approximately 280 mg/kg via a single i.p. injection. No mice, however, died from three daily injections of PENTO at 100 mg/kg/day. Furthermore, we observed that daily injections of PENTO at nontoxic doses over 3 days gradually improved the tumor pO_2 to about 17 mmHg (unpublished data, Lee I, 1990. 8). In the light of the fact that this drug is already used for other diseases[4~12], it appears that PENTO alone is potentially useful to increase the pO_2 and response of human tumors to radiotherapy.

Lee et al. also reported that NA increased blood flow and pO_2 in FSaII tumors of C3H mice[26]. As shown in Figure 5, the frequency distribution of measured tumor pO_2 had shifted to the right after treatment with PENTO+NA. Moreover, the percentage of regions with low oxygen tensions (below 2.5 mmHg) significantly decreased from about 28% to about 1% by PENTO+NA. PENTO has been reported to have a number of biological effects *in vivo*, which might influence the radiation damage[4~12]. However, our result indicates that an increase in intratumor pO_2 is the main mechanism for the increase in the radiation damage in tumors.

We concluded that a possible physiological mechanism in enhancement of *in vivo* radiosensitization by PENTO+NA appeared to be due mainly to an increase in tumor oxygenation. However, PENTO+NA did not increase the radiation-induced skin damage after local irradiation. Further studies using various concentrations of PENTO alone and in combination with NA to obtain better sequencing and maximal radiosensitization are warranted.

ACKNOWLEDGEMENTS

1. The authors wish to thank Mrs. Ae Lee for her help in the preparation of this manuscript. This work was supported by NCI grant number CA 13353.
2. Reprint requests to: Dr. Intae Lee.

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Pentoxifylline과 Nicotinamide의 병용에 의한 생체내 방사선 감수성 증강 효과

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조 문 준

Pentoxifylline (PENTO)는 적혈구의 유동성을 증가시켜 모세혈관의 적혈구 흐름을 증가시킨다. 또한 적혈구내 2,3-DPG를 증가시켜서 산소 친화력을 감소시켜 산소의 해리를 촉진시킨다. Nicotinamide (NA)는 종양내 혈류를 일시적으로 증가시켜서 종양내 급성 저산소 세포의 수를 감소시킨다. PENTO와 NA의 병용이 저산소 세포의 산소화에 의해서 방사선 감수성을 증가시킬 수 있는지를 확인하기 위하여 FSaII 생쥐의 섬유육종을 이용하여 실험을 시행하였다. 방사선에 의한 성장 장애가 유의하게 증가하였으며, 증가율은 2.5~2.8이었다. TCD_{50}가 대조 종양군에서는 57Gy였으나 PENTO+NA 투여 종양군에서는 32Gy로 1.8배의 TCD_{50}의 감소를 보였다. 정상피부의 방사선 감수성에는 영향이 없었다. PENTO+NA의 방사선 감수성의 증가를 규명하기 위하여 종양내 혈류의 변화, 종양내 산소농도를 laser Doppler flowmetry와 산소 미소전극 방법으로 측정하였다. PENTO+NA 투여후 10분 경과하여 혈류가 유의하게 증가하였으며 종양내 산소 분압도 8 mmHg에서 19 mmHg로 유의하게 증가함을 관찰하였다. 따라서 PENTO 또는 NA 단독보다 PENTO+NA 병용이 더욱 효과적이라 사료되며 생체내 종양의 방사선 감수성의 증가는 종양내 산소의 증가로 생각되며 더욱 방사선 감수성을 증가시키기 위하여 여러 농도의 PENTO의 단독 또는 NA와의 병용등에 대한 지속적인 연구가 필요하다.