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Effect of Tumor Hypoxia on Efficacy of Tirapazamine Combined with Fractionated Irradiation in Mouse Tumor

Il Han Kim, M.D.

Department of Therapeutic Radiology, Seoul National University College of Medicine, Seoul, Korea

Purpose : Tumor hypoxia can be overcome with hypoxic cytotoxin. In mouse tumor, tirapazamine's efficacy of the potentiating radiation effect was tested by the tumor oxygenation status combined with hyperfactionated radiotherapy.

Materials and Methods : The control and hypoxic mouse tumors were established by inoculation of RIF-1 tumor cells into the normal or previously irradiated back and thigh of C3H mice. When the tumors reached a proper size, both the control and hypoxic tumors were given hyperfractionated treatments (8 fractions/4 days) with saline (0.02 ml/g), tirapazamin (0.08 mM/0.02 ml/kg), irradiation (2.5 Gy), irradiation combined with tirapazamine given 30 minutes prior to each irradiation. The response was evaluated by the growth delay assay by measuring tumor size from day 0 (12 hrs prior to the first fractionation) to the day when the volume had 4-fold increase or cross sectional area had 2-fold increase.

Results : Overall growth pattern showed that tirapazamine potentiated radiation effect in back and thigh tumors grew in the normal and preirradiated tumor bed. With growth delay assay using reference point of initial tumor volume or cross sectional area, tirapazamine potentiated radiation effect 1.9 times for the control and 2.4 times for the hypoxic tumors in back, and 1.85 times for the control and 1.6 times for the hypoxic tumors. With reference of 4-fold increase of the initial volume or 2-fold increase of the cross sectional area, tirapazamine potentiated radiation effect 1.48 times for the control and 2.02 times for the hypxic tumors in back, and 1.85 times for the control and 1.6 times for the hypoxic tumors.

Conclusion : Present result indicated that radiation response of hypoxic tumors was potentiated by tirapazamine in the back or thigh tumors grew in the control or preirradiated tumor bed, and potentiation of the hypoxic tumors was equal to or greater than that of the control tumors in the back or thigh.

Key Words : Tumor hypoxia, Tirapazamine, Radiation, Growth delay

INTRODUCTION

Tumor cells under very low oxygen tension, that is, hypoxic cells were found to exist in almost all transplantable tumors in rodents.[1, 2] There is also evidence, both direct and indirect, for the presence of hypoxic tumor cells in a large proportion of solid tumors in human.[3~9] The existence of hypoxic clonogenic cells in solid tumors critically influences the response of tumors to and clinical outcome after radiotherapy[10~12] because it is well known that lethal effect of x- or γ-ray is so weak in hypoxic condition that its power is calculated as 2.5 to 3.0 in cell survival curves. This phenomenon of relative resistance of hypoxic cell to treatment was also found against some chemotherapeutic agents.[13]

Hyperbaric oxygen, transfusion of hemoglobin or artificial blood, hypoxic cell radiosensitizers, bioreductive hypoxic cytotoxins have been enthusiastically investigated in order to overcome the radioresistance by hypoxia. Tirapazamine (3-amino-1,2, 4-benzotriazine-1,4-dioxide), one of the most promising bioreductive hypoxic cell toxin, preferentially kills hypoxic cells *in vivo*[14, 15] and *in vitro*.[16] Combined with radiation or some anticancer chemotherapeutic agents, tirapazamine killed more cells than anticipated from simple addition of independent killing effects in some rodent tumors.[17, 18] Thus the hypoxic cells in certain tumors might not be obstacle for the local tumor control but can be

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Reprint requested to : Il Han Kim, M.D., Department of Therapeutic Radiology, Seoul National University Hospital
Tel : +82-2-760-2528 Fax : +82-2-765-3317
E-mail : ihkim@snu.ac.kr

exploited with hypoxic cytotoxins combined with fractionated radiotherapy.

As the phase II/III clinical trial of the drug is currently going on,[19] it is anticipated that hypoxic tumors might be more responsive than euoxic tumor to radiotherapy combined with tirapazamine. To test this, specific growth delay was measured after hyperfractionated irradiation combined with tirapazamine in tumors growing in unirradiated tissue and tumors growing in preirradiated tissue, that is more hypoxic resulted from the tumor bed effect,[20] using mouse RIF-1 tumors. SCCVII tumors were initially tried but soon abandoned because of its immunogenecity might disturb end points. Results show that hypoxic tumors were more responsive than control tumors to hyperfractionated irradiation combined with tirapazamine and thus suggest clinical implication of bioreductive radiotherapy for some hypoxic tumors if we can obtain oxygen profile before treatment, that is, effective non-invasive way of predictive assay for tumor oxygenation.

MATERIALS AND METHODS

1. Tumors and experimental animal

The RIF-1 sarcomas were maintained alternatively in vivo and *in vitro*. The female C3H/Km mice were bred and kept in filter-top cage during experiments under defined flora condition in the Stanford Research Animal Facility. Mice were 12~16 weeks old and weighed 25~35 g. The derivation of the cell lines and details of handling have been described[21~23]. Tumors cells were harvested from monolayer culture and 2×10^5 tumor cells in 0.05 ml of Waymouth's media with 15% fetal calf serum were inoculated in the lower back intradermally or thigh of right hindleg intramuscularly. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (67.5 mg/kg) before inoculation of tumor cells.

2. Tumor models

Tumors growing in preirradiated tissue (hypoxic tumors) and tumors growing in normal tissue (control tumors) were used in this study. The hypoxic tumors usually have higher fractions of hypoxic fraction than the control tumors because of the tumor bed effect. The profiles of tumor hypoxia and details of establishing these hypoxic tumor models were described previously.[24] In brief, the tumor beds e.g., lower back or thigh of right hindleg of the mice were irradiated (20 Gy single fraction) 4 wks before inoculation of tumor cells, to establish the hypoxic tumors. For the irradiation of tumor bed the unanesthetized mice were placed in individual lead jigs with a cut-out to enable the tumor bed to be irradiated without irradiation of the rest of the mouse. Irradiation was done using a 250 kVp X-ray apparatus (Philips RT 250; 15 mA with 0.35 mm Cu filter, SSD of 31 cm) at a dose rate of 1.69 Gy/min. No specific treatments were applied to the tumor beds of the control tumors before inoculating tumor cells. In RIF-1 tumors growing in thigh of right hindleg, the median tumor pO_2 determined by a computerized polarographic microelectrode system (Sigma-pO_2-Histograph, Eppendorf, Germany) was 11.8 mmHg in the control tumors, and 8.5 mmHg in the preirradiated tumors.

Fractionated treatments were performed when volumes of back tumors reached about 100 mm^3 or cross-sectional areas of thigh of hindleg reached about 130 mm^2. Volumes (V) of back tumors were calculated by an ellipsoid approximation using the 3 orthogonal diameters a, b, and c ($V=abc\pi/6$) and cross-sectional areas (A) of thigh tumors were calculated using a long diameter (l) and a diameter (p) perpendicular to it in cross-sectional plane ($A=lp\pi/4$). Measured values were corrected for skin thickness.

3. Tirapazaime, a hypoxic cytotoxin

Tirapazamine (3-amino-1,2,4-benzotriazine-1,4-dioxide : MW= 179.95) was synthesized in SRI International (Menlo Park, U.S.A.). The drug was freshly dissolved in physiological saline and injected intraperitoneally in a volume of 0.02 ml/g of body weight at a dose of 0.08 mM/kg (14.9 mg/kg). Ultrasonication was applied for the rapid solution sometimes.

4. Irradiation

Unanesthetized mice were placed in individual lead jigs with a cut-out to enable the whole tumor on the back or in the thigh of the hindleg to be irradiated while protecting the rest of the mouse body. Irradiation conditions were the same as those used for irradiation of tumor beds before inoculation.

5. Experimental scheme of hyperfractionation

Each group of tumor (5 mice per group) were treated with four arms of treatments, saline (0.02 ml/g) as the control, tirapazamine, irradiation, and irradiation combined with tirapazamine. Each arm was delivered by hyperfraction-

ation schedule of 8 fractions in 4 days. Trapazamine was injected 30 min prior to each irradiation in combination arm. Each fractionation schedule consisted of twice-a-day treatment with an interfraction interval of 12 hrs. The dose of tirapazamine per fraction was 0.08 mM/kg and radiation dose was 2.5 Gy per fraction. The mice of control or tirapazamine alone group were kept in mice-jig without irradiation during same time as irradiation group. Saline (0.02 ml/g) was intraperitoneally injected to the irradiation alone group, too.

5. Evaluation of tumor response

Tumor response was evaluated by growth delay assay. The size of the RIF-1 tumors in either back or thigh, were measured 12 hrs before treatment and 3 times a week during and after treatment. Tumor volumes were calculated for the back tumors and cross sectional areas for the thigh tumors. Median volumes or median cross sectional areas were obtained from five independently assayed tumors at each point to get a overall tendency.

The size of back tumors were measured until its volume reached 4 times of the initial (12 hrs before the first fractionated treatment), and size of thigh tumors were measured until its cross sectional area reached 2 times of the initial. Growth delay (GD) was defined as the time needed after treatment to reach to the initial size at time 0 (12 hrs before the first fractionated treatment) or to 4-fold increase in volume or 2-fold increase in cross sectional area. Growth delay of each tumor was obtained by regression of tumor growth in graph in which data were plotted after logarithmic conversion. Specific growth delay (SGD) of each tumor was calculated by the following equation;

$$\text{Specific growth delay (SGD)} = \frac{\text{Gowth Delay (GD)}}{\text{Doubling Time (DT)}}$$

DT denotes here mean of doubling time for volume or cross sectional area of saline treated back or thigh tumors, either hypoxic or control.

RESULTS

1. Overall growth pattern

The mean of adjusted volume of back tumors at day 0 (12 hrs prior to the first fractionated treatment) were 106± 20 mm3 at the preirradiated tumor bed and were 100±19 mm3 at the normal tumor bed. The mean of adjusted cross sectional of the thigh tumors at day 0 were 129±20 mm^2 at the preirradiated tumor bed and were 137±16 mm^2 at the control tumor bed. The volume of the back tumors was measured until day 6 (saline treatment as control) to 20 (irradiation and tirapazamine) for the normal tumors at back or until day 22 to 37 for the hypoxic tumors. The cross sectional area of the thigh tumors was measured until day 14 (saline treatment as control) to 37 (irradiation and

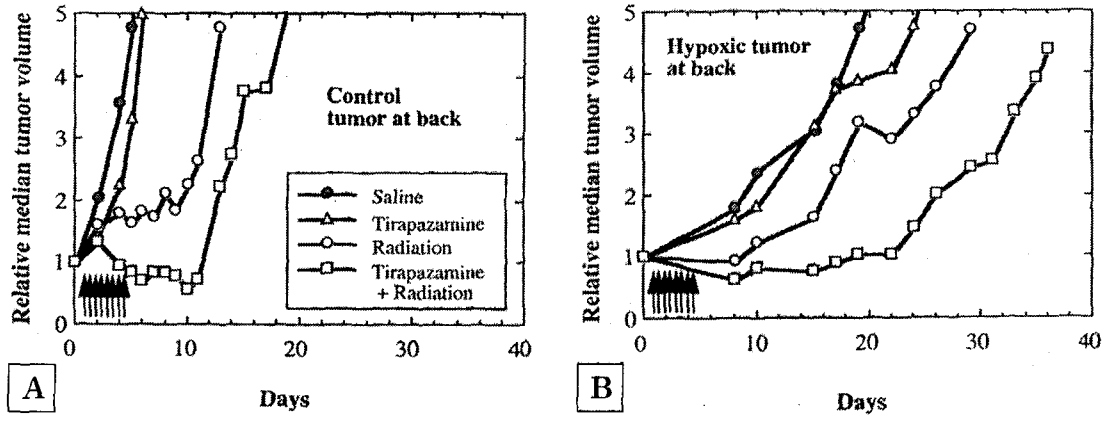

Fig. 1. Overall growth pattern of RIF-1 tumor in the back of C3H mice after hyperfractionated treatment (8 fractions/4 days, from day 1 to 4) with irradiation (2.5 Gy), tirapazamine (0.08 mM/kg), irradiation combined with tirapazamine, and saline (0.02 ml/g) as the control was shown as a function of time. The volume of tumors growing in unirradiated or preirradiated back (Panel A and B) was measured until its volume reached 4 times of the initial (12 hrs before the first fractionated treatment). Each arrow denotes fractionated treatment and each point shows median of 5 tumors from independent mice. It was definite from this data that tirapazamine potentiated radiation response in tumors of the back growing in the control or preirradiated tumor bed.

tirapazamine) for the control tumors to day 16 to 51 for the hypoxic tumors of the thigh.

The overall tumor growth pattern which was manifested as the median tumor volume or cross sectional area relative to the day 0 measurement of each treatment group in each type of tumor was shown in Fig. 1A, 1B, 2A, and 2B. As expected, tumor grew slowly in the preirradiated bed compared to the control bed. It was clear that tirapazamine definitely potentiated radiation effect in back and thigh tumors grew in the normal or preirradiated bed.

2. Growth delay

Geometric mean of growth delay and specific growth delay calculated from growth curves of individual tumors were shown for each group.

When the reference point was the initial volume or cross sectional area(Table 1), tirapazamine alone produced absolute d specific growth delay of 0.7 to 0.9 for the control and hypoxic tumors in back. But when tirapazamine was combined with hyperfractionated irradiation higher enhancement of absolute or specific growth delay was produced, approximately 1.9 times for the control and 2.4 times for the hypoxic tumors than that of radiation alone. In the thigh, tirapazamine produced absolute growth delay of 2.4 to 5.9 days and specific growth delay of 0.4 to 0.5 for control and hypoxic tumors. The combination of tirapazamine with irra-

Table 1. Growth Delay after Hyperfractionated Treatments (8 fractions/4 days) with Tirapazamine (0.08 mM/kg), Irradiation (2.5 Gy), and Irradiation Combined with Tirapazamine in Comparison with the Control (saline 0.02 ml/g) in RIF-1 Sarcoma. Each Data of Independent Tumor Was Regressed to Get Growth Delay and Its Logarithmic Mean Value was Obtained

Tumor bed	Treatment	Growth delay-0[§]	
		Absolute (d)	Specific
Normal back	TPZ*	1.81±0.18[‖]	0.86±0.09
	IR[†]	6.33±0.42	3.00±0.21
	TPZ+IR[†]	12.10±0.70	5.73±0.35
Preirradiated back	TPZ	5.20±0.85	0.73±0.14
	IR	8.04±0.53	1.13±.0.13
	TPZ+IR	19.43±1.60	2.73±0.35
Normal thigh	TPZ	2.44±0.76	0.40±0.13
	IR	8.04±0.81	1.30±0.15
	TPZ+IR	14.87±0.54	2.41±0.18
Preirradiated thigh	TPZ	5.86±0.58	0.52±0.14
	IR	12.81±0.28	1.07±.0.27
	TPZ+IR	19.44±1.81	1.72±0.46

*Tirapazamine
†Irradiation
†Tirapazamine was intraperitoneally injected 30 min prior to each irradiation.
§with reference of day 0 volume or cross sectional area
‖Mean±S.D.

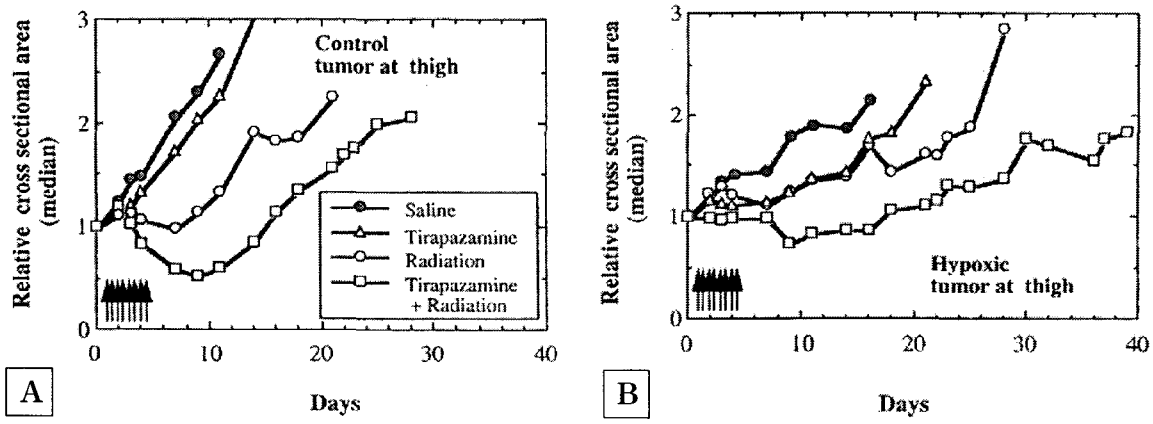

Fig. 2. Overall growth pattern of RIF-1 tumor in the thigh of C3H mice after hyperfractionated treatment (8 fractions/4 days, from day 1 to 4) with irradiation (2.5 Gy), tirapazamine (0.08 mM/kg), irradiation combined with tirapazamine, and saline (0.02 ml/g) as the control was shown as a function of time. The cross sectional area of tumors growing in unirradiated or preirradiated thigh (Panel A and B) was measured until its cross sectional area reached 4 times of the initial (12 hrs before the first fractionated treatment). Each arrow denotes fractionated treatment and each point shows median of 5 tumors from independent mice. It was definite from this data that tirapazamine potentiated radiation response in tumors of the thigh growing in the control or preirradiated tumor bed.

diation produced a equivocal enhancement of absolute or specific growth delay of approximately 1.85-fold longer for control and 1.6-fold longer for hypoxic tumors than that of irradiation alone.

When growth delay was calculated with reference of increase to 4-fold of the initial volume or 2-fold of cross sectional(Table 2), tirapazamine produced absolute specific growth delay of 0.2 to 0.7 for the control and hypoxic tumors in back. But when tirapazamine was combined with hyperfractionated irradiation higher enhancement of absolute or specific growth delay was produced, 1.48 times for the control and 2.02 times for the hypoxic tumors than that of radiation alone. In the thigh, tirapazamine produced absolute growth delay of 2.0 to 8.5 days and specific growth delay of 0.24 to 0.59 for control and hypoxic tumors. The combination of tirapazamine with irradiation produced a equivocal enhancement of absolute or specific growth delay of approximately 1.85-fold longer for control and 1.6-fold longer for hypoxic tumors than that of irradiation alone.

Table 2. Growth Delay after Hyperfractionated Treatments (8 fractions in 4 days) with Tirapazamine (0.08 mM/kg), Irradiation (2.5 Gy), and Irradiation Combined with Tirapazamine in Comparison with the Control (saline 0.02 ml/g) in RIF-1 Sarcoma. Each Data of Independent Tumor was Regressed to Get Growth Delay and Its Logarithmic Mean Value was Obtained

Tumor bed	Treatment	Growth delay-G[§]	
		Absolute (d)	Specific
Normal back	TPZ*	1.38±0.37[‖]	0.66±0.18
	IR[†]	8.39±0.74	4.00±0.36
	TPZ+IR[†]	12.45±1.45	5.93±0.70
Preirradiated back	TPZ	2.52±2.12	0.28±0.24
	IR	9.21±1.28	1.03±0.16
	TPZ+IR	18.54±1.31	2.08±0.20
Normal thigh	TPZ	2.01±1.00	0.24±0.12
	IR	10.88±1.77	1.32±0.23
	TPZ+IR	18.40±2.62	2.23±0.35
Preirradiated thigh	TPZ	8.50±3.20	0.59±0.22
	IR	14.72±2.77	1.02±0.19
	TPZ+IR	28.02±3.42	1.95±0.25

*Tirapazamine
†Irradiation
†Tirapazamine was intraperitoneally injected 30 min prior to each irradiation.
§with reference of increase to 4-fold of the initial volume or 2-fold of the initial cross sectional area
‖Mean±S.D.

Discussion

This study confirmed that radiation effect was potentiated by tirapazamine, a hypoxic cytotoxin, in mouse tumor gre in the control bed and preirradiated hypoxic bed. Response of some transplantable mouse tumors to irradiation was enhanced when irradiation was combined with hypoxic cytotoxin which preferentially kills hypoxic cells.[25] The present studies demonstrated after rather long-term observation that response of hypoxic tumors to fractionated irradiation was not worse but equally or greatly potentiated than euoxic tumors of same size and histology when combined with a hypoxic cytotoxin, tirapazamine.

It seemed that higher enhancement in the preirradiated bed in comparison with the normal rumor bed depended upon the tumor inoculation site. This higher enhancement was more evident in the back tumors than in the thigh rumors. But how can the above be explained in RIF-1 tumors after fractionated irradiation combined with tirapazamine?

There can be some differences between two kinds of tumor model. Firstly for the establishment of 'tumor bed effect', the back was irradiated tangentially, that is, only the epidermis and some portion of dermis were irradiated but deep structures were lead-shielded, while the whole circumference and whole length of the right hidleg was included in the radiation field four weeks before inoculation. Secondly tumor cells were inoculated subcutaneously in the back but intramuscularly in the hindleg. Thirdly we had a solid evidence that the measured volume of back tumor represent the real tumor volume because we observed sharp margin of the back tumor from many cases of excised tumors. But there simple measurement of cross sectional area rather than volume of the hindleg tumors might not represent the true tumor volume because of ambiguity of proximal or distal boundary. Lastly two tumors differed in the pattern of vascular supply, that is, the back facia seemed to be main and limited vascular supplier to the back tumor but the hindleg tumor seemed to have large number of supplying vessels because tumor grew in the middle of hindleg muscles. But likewise in tumors grew in the preirradiated bed, it is possible that relatively large proportion of vascular network was damaged heavily. The large portion of thigh tumors especially ones grew in the preirradiaetd tumor bed showed swelling, necrosis, or amputation of the part hidleg.

Next, as already known, RIF-1 tumor has some unique features in the aspects of tumor oxygenation. Firstly there is no increase in the radiobiologically hypoxic fraction as the size of the tumor increases from 100 mm^3 to 400 mm^3 contrary to the other mouse tumors.[21, 24, 26] Secondly, there is discrepancy between radiobiologically hypoxic fraction and pO_2 or oxyhemoglobin saturation status,[24, 27] while there exists correlation between these parameters in SCCVII tumors. Thirdly hypoxic RIF-1 tumor did not show greater potentiation than the control tumors after treatment of fractionated irradiation and tirapazamine using *in vivo-in vitro* assay.[28] The reason for this discrepancy in RIF-1 tumors cannot be explained at present.

The present results suggest that the difference in tumors grew in different anatomic site might result in the difference in the potentiation of radiation response to fractionated irradiation combining with tirapazamine. But in overall, important thing is that radiation response of hypoxic tumors can be as at least equal as that control euoxic tumors after combining with tirapazamine.

If the fraction of hypoxic cells in the human solid tumor reestablish itself to the pretreatment levels (termed as rehypoxiation) after each dose of tirapazamine as we have demonstratedin the SCVII tumors,[14] it can be clinically implicated that hypoxic tumors are not radioresistant obstacle any more or can become more responsive than less hypoxic tumors by conventionally fractionated irradiation combined with hypoxic cytotoxic agents.

In conclusion, this study has demonstrated that a hypoxia selective cytotoxin tirapazamine potentiated cell killing by hyperfractionated irradiation in mouse tumor model and its potentiation effect might be equal or greater in hypoxic tumor than the control tumors.

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국문 초록

마우스종양에서 분할방사선조사와 병용된 Tirapazamine의 효과에 미치는 종양 저산소상태의 영향

서울대학교 의과대학 치료방사선과학교실

김 일 한

목 적 : 종양내 저산소상태는 저산소세포치사제에 의하여 극복이 가능하다. 방사선의 반응을 증강시키는 tirapazamine 의 효과가 마우스 종양에서 분할방사선조사와 병용된 상태에서 종양내 산소상태에 따라 어떠한 영향을 받는가를 확인하고자 하였다.

대상 및 방법 : 대조군 및 저산소 상태의 종양을 수립하기 위하여 정상 및 4주전에 방사선 조사를 받았던 마우스의 등과 하지에 RIF-1 종양을 이식하였다. 종양이 일정한 크기에 도달하면, 대조군 및 저산소 상태 종양에 대하여 생리식염수(0.02 ml/g), tirapazamine (0.08 mM/kg), 방사선조사(2.5 Gy), tirapazamine 과 방사선조사의 병용 등을 사용하여 4일간 8회의 과분할 치료를 시행하였다. 등의 종양 체적이 4 배로 증가하거나 하지의 종양 단면이 2배로 증가할 때 까지 종양의 크기 변화를 측정하여 얻은 종양성장의 지연을 기준으로 각 치료에 대한 반응을 평가하였다.

결 과 : 등 및 하지의 정상 및 저산소종양의 평균증식양상으로 부터 tirapazamine이 방사선의 반응을 증강시켰음을 알 수 있다. tirapazamine에 의한 방사선증강효과는 초기의 종양체적 또는 종양단면적을 기준으로 한 증식지연실험 결과 등의 정상종양에서는 1.9배, 저산소종양에서는 2.4배였으며 하지의 정상종양에서는 1.85배, 저산소종양에서는 1.6배였다. 초기종양체적의 4배 증식 또는 종양단면적의 2배 증식까지의 기간을 기준으로 설정한 증식지연실험결과 등의 정상종양에서는 1.48배, 저산소종양에서는 2.02배였으며 하지의 정상종양에서는 1.85배, 저산소종양에서는 1.6배였다.

결 론 : 분할 방사선조사에 tirapazamine을 병용할 경우 방사선조사효과는 증강되며, 저산소상태에 있는 마우스 종양에서의 증강효과가 대조 종양에서의 증강효과와 동일하거나 더욱 양호할 가능성을 제시할 수 있었다.

핵심용어 : 종양의 저산소상태, tirapazamine, 방사선분할조사, 종양증식지연