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The Effect of Gingko Biloba Extract on Energy Metabolic Status in C3H Mouse Fibrosarcoma : Evaluated by in vivo ^{31}P Magnetic Resonance Spectroscopy

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Purpose : Gingko biloba extract (GBE), a natural product extracted from Gingko leaves, is known to increase the radiosensitivity of tumors. This radiosensitization probably arises from the increase in the peripheral blood flow by decreasing the blood viscosity and relaxing the vasospasm. The influence of a GBE on the metabolic status in fibrosarcoma II (FSaII) of a C3H mouse was investigated using ^{31}P magnetic resonance spectroscopy (MRS).

Materials and Methods : Eighteen C3H mice with fibrosarcoma II (from 100 mm^3 to 130 mm^3) were prepared for this experiment. The mice were divided into 2 groups; one (9 mice) without a priming dose, and the other (9 mice) with a priming dose of GBE. The GBE priming dose (100 mg/kg) was administered by an intraperitoneal (i.p.) injection 24 hours prior to the measurement. First ^{31}P MRS spectra were measured in the mice from each group as a baseline and test dose of GBE (100 mg/kg) was then administered to each group. One hour later, the ^{31}P MRS spectra were measured again to evaluate the change in the energy metabolic status.

Results : In the group without the priming dose, the mean pH, PCr/Pi, PME/ATP, Pi/ATP, PCr/(Pi+PME) values 1 hour after the test dose were not changed significantly compared to the values at the baseline. However, in the group with the priming dose, the mean PCr/Pi, Pi/ATP, PCr/(Pi+PME) values 1 hour after the test dose changed from the baseline values of 0.49, 0.77, 0.17 to 0.74, 0.57, 0.28 respectively. According to the paired t-test, the differences were statistically significant.

Conclusion : The above findings suggest that the metabolic status is significantly improved after administering GBE if the priming dose is given 24 hours earlier. This shows that the radiosensitizing effect of GBE is based on the increase of tumor blood flow and the improvement in the metabolic status.

Key Words : Gingko biloba extract, Mouse fibrosarcoma, Energy metabolism, ^{31}P magnetic resonance spectroscopy

Introduction

The rapid growth of malignant tumor beyond the perfusion range of blood vessels and abnormal vasculature in tumors increase hypoxic area.[1] This metabolically depleted hypoxic cells comprise 10~15% of tumor cells and are less sensitive to radiation.[2,3] Thus hypoxic cell is thought to be one of the major causes of failure after radiotherapy. Many new approaches, including blood flow modifiers, artificial oxygen carriers, oxygen releasing chemicals and inhibitors of oxygen consumption, are currently being explored to improve the results of radiotherapy through hypoxic cell radiosensitization. Recently considerable interest has been shown in the physiological manipulation of tumor oxygenation by the systemic administration of vasoactive agents.[4] But their clinical

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efficiency is not clear until now. Extracts from the leaves of Ginkgo biloba have been used therapeutically for centuries utilizing its vasoregulating activity of arteries, capillaries, veins. Main action of Ginkgo biloba extract (GBE) is known to induce the increase in the peripheral blood flow by decreasing blood viscosity and relaxation of vasospasm.[5] Our previous data suggested that GBE is effective in increasing the antitumor effect of radiation by decreasing tumor hypoxia, [6~8] and this is probably by increasing the tumor blood flow.

^{31}P magnetic resonance spectroscopy (MRS) is a non-invasive analytical tool currently being studied extensively to measure the amount of hypoxic cell by using energy metabolism in vivo. ^{31}P MRS provides spectra with fairly well separated resonances of important phosphorus metabolites, phosphomonoester (PME), inorganic phosphate(Pi), phosphodiester (PDE), creatine phosphate (PCr), and 3 levels of adenosine triphosphate (α-ATP, β-ATP, γ-ATP). Recent technical advances on ^{31}P MRS have promoted the study of metabolic change induced by vasoactive agents; agents (eg. hydralazine) that decrease blood flow produce a decrease in high energy phosphates measured by ^{31}P MRS, whereas agents (eg. flunarizine) that increase blood flow produce the opposite effect.[4] The influence of GBE on metabolic status in fibrosarcoma (FSaII), growing in the dorsum of hind foot in C3H mouse, was investigated using ^{31}P MRS. Practically the hypothesis tested was that energy status of tumor would be improved by test dose of GBE given i.p. one hour before ^{31}P MRS with or without priming dose given 1 day earlier.

Methods and Materials

1. Tumor transplantation

Eight to 14 week old C3H/Sed mice weighing 20~30 g were used for this study. This mice were obtained from Edwin L Steel laboratory of Department of Radiation Medicine, Havard Medical School, Massachusetts General Hospital and were bred and maintained in laminar flow clean animal room in Radiation Biology Laboratory of Cancer Research Center, Seoul National University Medical College. These mice have only 4 defined flora of Clostridium (C.356, C. inoculum, C. bareki, C. clostridiformis) and have no virus and no bacteria.[6] Fifth generation isotransplants of the spontaneous fibrosarcoma, FSaII (poorly differentiated fibrosarcoma) were used throughout these experiments. FSaII is very weakly immunogenic.[9] Tumor material for inoculation was obtained by sterile dissection of flank tumors. Macroscopically viable tumor tissue was minced into fine pieces and single cell suspensions were prepared by trypsinization. Viable tumor cell number was counted by hemocytometer using typhan blue exclusion method[10] and were diluted appropriately in Hanks' balanced salt solution (HBSS) for adjustment of cell count. One thousand viable tumor cells mixed with lethally irradiated (120 Gy in vitro) 2×10^5 tumor cells were transplanted into the dorsum of hind foot in an inoculum volume of 5 microliter. After transplantation, tumor grew in every mouse and over 90% of the tumors reached 6 mm on day 10 after transplantation. The 3 perpendicular tumor diameters were measured with Vernier caliper. Tumor volmes were calculated from measurements of length (R_1) width (R_2) and heigh t(R_3) on the assumption that the tumors were hemi-ellipsoids.

$$V=\frac{4}{3}\pi\times\frac{R_1}{2}\times\frac{R_2}{2}\times\frac{R_3}{2}$$

The tumors at the volume of 100~130 mm^3 were used for this experiment of ^{31}P MRS measurement.

2. ^{31}P MRS experiment

Each mouse was restrained within an individual cage specially designed for immobilization, with tumor bearing foot protruding through an opening of the cage. The cage fit the body of the mouse snugly and allowed little room for movement. Ventilation was provided by perforations on the wall of the cage. The mouse was immobilized by taping tail to a bottom of a cage and tumor bearing foot was secured to a plastic board. This preparations provided satisfactory

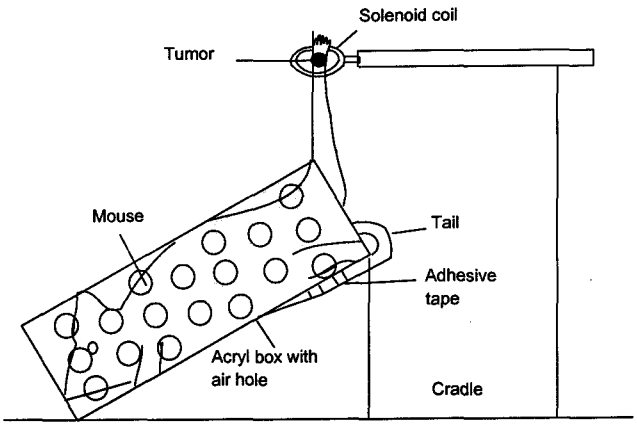

Fig. 1. Schematic representation of the mouse immobilization setup for MRS measurements.

immobilization of the tumor bearing foot throughout MRS data acquisition (approximately 40 min) (Fig. 1).[11]

^{31}P MRS experiments were performed on a 4.7 Tesla/30 cm bore magnet Biospec MRS/MRI system (Bruker, Switzland) and MRS signals were obtained by using the 2 turn, 1 cm inner diameter, solenoid coil which was placed directly over the tumor bearing foot. Solenoid coil was double tuned at 200.215 MHz of ^{1}H resonance frequency and at 81.037 MHz of ^{31}P resonance frequency.[12, 13] Once the animal was positioned in the bore of the magnet, magnetic static field (B_0) homogeneity was optimized by shimming on the in vivo water ^{1}H resonance signal of the tumor prior to the spectral accumulation. The typical acquisition parameters chosen to optimize sensitivity were as follows: spectral width 5,000 Hz, block size 8 K, interpulse delay 2.2 sec, pulse width 40 μs, acquisition 1024, total average time 38 min/spectrum. The size of spectral peaks was measured by area integration method and spectral changes expressed as the ratio of the peak areas. In this study, the relative concentrations of phosphomonoester (PME), inorganic phosphate (Pi), creatine phosphate (PCr), adenosine triphosphate (ATP) and pH have been evaluated as spectral parameters. PCr was used as an internal chemical shift reference (as signed as 0.00 ppm). Intracellular pH was calculated from the chemical shift difference between PCr and Pi using appropriate form of the Henderson-Hasselbach equation.

3. Experimental groups

Ginkgo biloba extract (GBE, Sunkyong industry) containing 24% of ginkgoflavonoglycosides and 6% of terpenoids was used. GBE was dissolved in distilled water to final concentration of 10 mg/ml. Total 18 mice with small tumors were prepared for this experiment and divided evenly into two groups; one group without priming dose (9 mice), the other group with priming dose (9 mice). The priming dose of GBE (100 mg/kg, dissolved in D/W) was administered intraperitoneally 24 hours before ^{31}P MRS measurement. First, ^{31}P MRS measurement were carried out on separate groups of mice to obtain baseline spectra and then test dose (100 mg/kg) of GBE was administered to each group. One hour later, ^{31}P MRS spectra measurement was repeated to evaluate change of energy metabolic status. For the statistical comparison of spectral parameters between before and after test dose in each group, paired t-test was performed. Significance is accepted for $p<0.05$.

Results

The effect of GBE on ^{31}P MRS spectra in each group is given in Fig 2. and the changes of spectral parameters after test dose of GBE are shown in Fig 2~6. In priming(-)

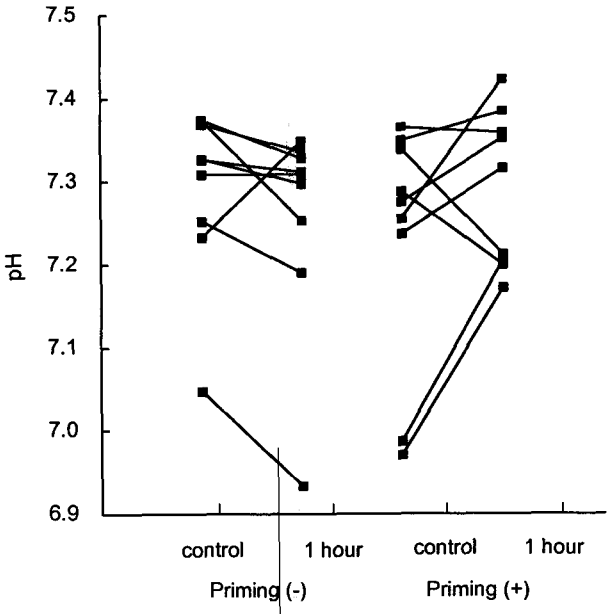

Fig. 2. The effect of GBE on pH measured by ^{31}P MRS in each group.

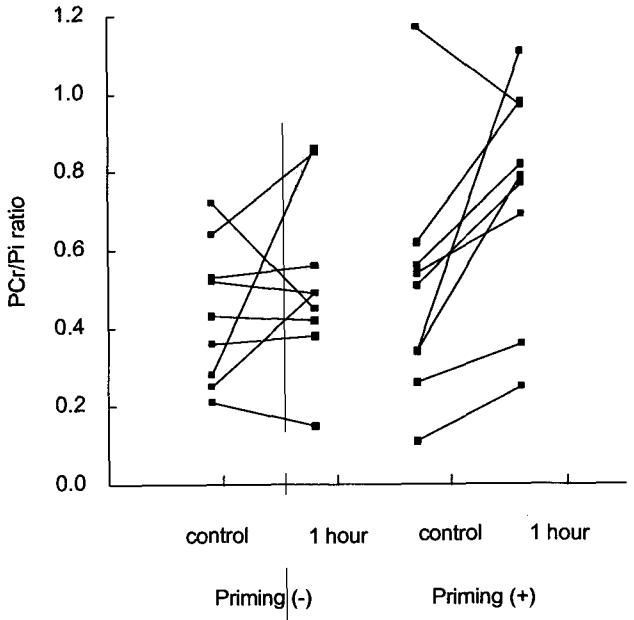

Fig. 3. The effect of GBE on PCr/Pi measured by ^{31}P MRS in each group.

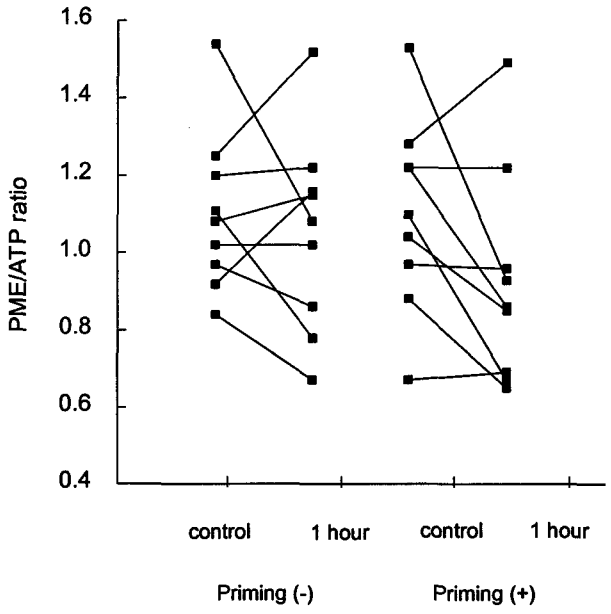

Fig. 4. The effect of GBE on PME/ATP measured by ^{31}P MRS in each group.

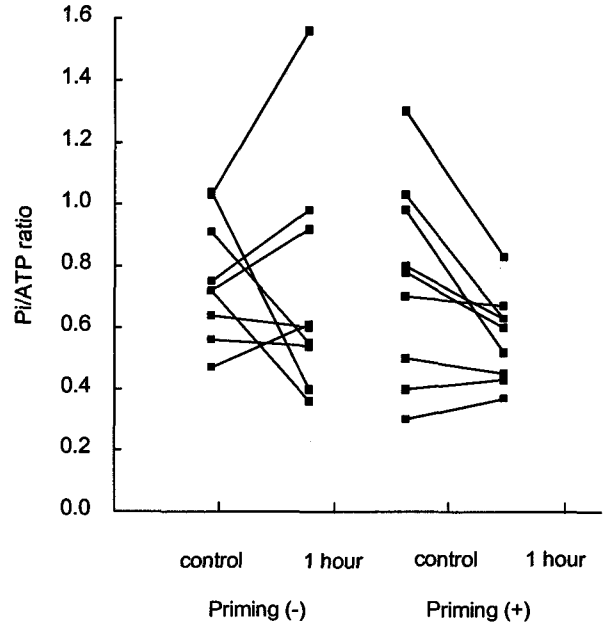

Fig. 5. The effect of GBE on Pi/ATP measured by ^{31}P MRS in each group.

group, the mean pH, PCr/Pi, PME/ATP, Pi/ATP, PCr/(Pi+PME) value at baseline was 7.29, 0.44, 1.10, 0.76, 0.18. At the examination 1 hour after test dose (100 mg/kg), the corresponding value were 7.26, 0.52, 1.05, 0.72, 0.19 respectively. There was slight improvement of metabolic status but

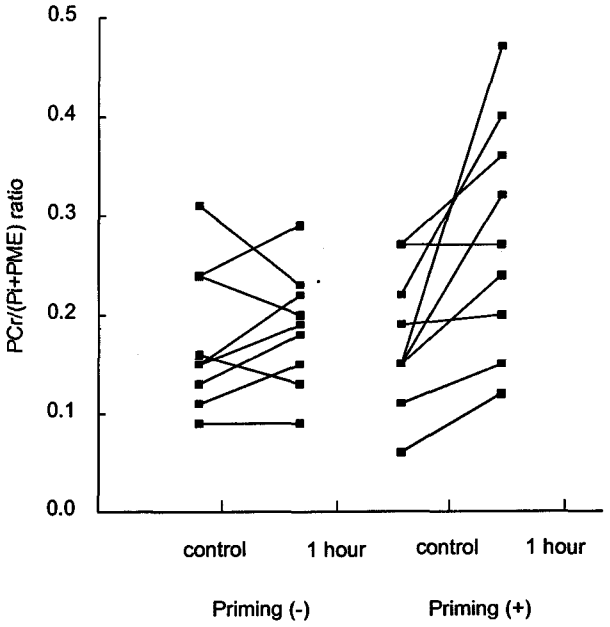

Fig. 6. The effect of GBE on PCr/(Pi+PME) measured by ^{31}P MRS in each group.

according to paired t-test, the differences were not statistically significant (Table 1). In priming(+) group, the mean pH at the baseline was 7.23 compared with values of 7.29. at 1 hour after test dose. According to paired t-test, the difference was not statistically significant (p=0.18). The mean PCr/Pi of 0.49 was recorded at the baseline and increased to 0.74 at 1 hour after test dose. The increase in PCr/Pi was by 51% and the difference was statistically significant (p= 0.02). The mean PME/ATP of 1.1 was recorded at the baseline and decreased to 0.92 at 1 hour after test dose. The decrease in PME/ATP was by 16% and the difference was borderline significant (p=0.07). The mean Pi/ATP was 0.77 at the baseline and decreased to 0.57 at 1 hour after test dose. The decrease in Pi/ATP was by 26% and the difference was statistically significant (p=0.02). The mean PCr/(PME+Pi) was 0.17 at the baseline and increased to 0.28 at 1 hour after test dose. The increase in PCr/(PME+Pi) was by 65% and the difference was statistically significant (p=0.01) (Table 1). These findings indicate that the metabolic status is improved slightly after a single administration (in priming(-) group) and significantly after repeated administration (in priming (+) group) of GBE.

Table 1. Effects of GBE on Metabolic Parameters

Parameters	Priming (-)		p^*	Priming (+)		$p^\dagger$
	Control	1 hour		Control	1 hour	
PH	7.29±0.03[†]	7.26±0.04	0.18	7.23±0.05	7.29±0.03	0.18
PCr/Pi	0.44±0.06	0.52±0.07	0.35	0.49±0.10	0.74±0.10	0.02
PME/ATP	1.10±0.07	1.05±0.09	0.54	1.10±0.08	0.92±0.09	0.07
Pi/ATP	0.76±0.07	0.72±0.13	0.78	0.77±0.11	0.57±0.05	0.02
PCr/(Pi+PME)	0.18±0.02	0.19±0.02	0.53	0.17±0.02	0.28±0.04	0.01

*paired t-test comparison in priming (-) group
†paired t-test comparison in priming (+) group
†mean±standard error

Discussion

Ginkgo biloba extract has been used for treatment of peripheral vascular occlusive disease such as intermittent claudication and cerebral insufficiency in European countries for over 20 years. The effectiveness of GBE in improving peripheral vascular circulation has been proven in many laboratory and clinical studies and side effects reported were very rare.[5] The effects of GBE may be caused by single active ingredients or by the combined action of the many active agents found in the extracts. The most important substances of GBE consists of ginkgo-flavone glycosides (flavonoids) and terpenoids (ginkgolides and bilobalides). GBE which used in this experiment comprised of 24% of flavonoids and 6% of terpenoids. GBE exerts positive effects on blood flow because of its hemorrheological actions and relief of vasospasm. In vitro and ex vivo study, GBE increased red blood cell deformability and decreased red blood aggregation. For example, 35 or 70 μg/ml solutions significantly reduced erythrocyte aggregation in blood samples from 20 patients with cerebral or peripheral vascular disease[14] and whole blood viscosity were reduced by GBE in patients with generalized arteriosclerotic lesions.[15] Blood flow in the nail fold capillaries, measured using nail fold capillaroscopy, also increased significantly by about 57% ($p<0.004$) 1 hour after GBE administration.[16] The overall vascular actions of GBE involve a maintenance of arterial and venous tone (beneficial in a hypoxic area with vasomotor paralysis), combined with an arterial relaxing effect, which could theoretically offset vascular spasm. Importantly, blood flow was increased in the damaged ischemic region as well as the healthy surrounding tissue - in other words, the extract was not associated with a steal phenomenon.[17, 18]

When the tumor volume grows larger, hypoxic cell fraction in the tumor increases. It has been established that the presence of this hypoxic cells in solid tumors influences their response to radiotherapy and chemotherapy and may limit their curability by these treatment modalities. Above pharmacological action of GBE suggest the possibility that GBE may be able to increase tumor blood flow and improve supply of oxygen and nutrition to the tumor cells which are in hypoxic and nutrient deprived status and thus enhance sensitivity to radiation. Our previous study was performed to test the effect of GBE on radiosensitivity of tumor and normal tissue and estimate the change of hypoxic fraction in tumor using fibrosarcoma (FSaII) growing in leg muscle of C3H mice. The results have already showed that the effect of radiation is slightly increased by administration of a single dose of GBE, but markedly after two doses as measured by tumor growth delay, tumor control dose and hypoxic fraction changes.[6, 7] Tumor growth time to reach a tumor volume from 100 mm^3 to 500 mm^3 was measured. Radiation (10 Gy) tumor growth delay was elongated to 1.23 times by a single dose of 50 mg/kg of GBE given 1 hour before irradiation and 1.49 times by 100 mg/kg of GBE. These elongations were not statistically significant. When priming dose (100 mg/kg) was given 24 hours before test dose (100 mg/kg), radiation tumor growth delay was further elongated, 1.84 times. This elongation was statistically significant. Radiation dose for 3 day tumor growth delay was 12.44 Gy for radiation alone and 6.11 Gy for two doses of GBE plus radiation, with enhancement ratio of 2.04.[6] $TCD_{50/120}$'s were 81.7 (95 % C.I.; 77.7~86.0) Gy when irradiated under clamp hypoxia, 69.6 (66.8~72.5) Gy under air, 67.5 (64.1~71.1) Gy with a single dose, and 62.2 (59.1~65.5) Gy when two doses of 100 mg/kg of GBE were given before radiation. The 95% confidence intervals of

$TCD_{50/120}$'s did not overlap between radiation alone and two doses of GBE plus radiation group. The hypoxic fraction, estimated from the $TCD_{50/120}$ data, decreased by administration of GBE, from 10.6% for control to 7.2% after single dose and to 2.7% after two doses of 100 mg/kg GBE.[7] Radiation effect on normal tissue, estimated by acute skin reaction and jejunal crypt survival assay, was not changed by GBE.[19]

According to above in vivo studies on tumor bearing animals, we planned this study to determine whether GBE can bring about changes in tumor metabolism detected by ^{31}P MRS, and if so, whether these changes are consistent with the alterations demonstrated by radiobiological endpoints.

The foot pad was chosen for tumor implant to reduce spectral contamination from nearby tissues, specifically muscle may contaminate the spectra of tumors under 100 mm^3 when other implantation sites are employed.[20] In this experiment, a spectrum of the tumor free foot demonstrates the absence of any mobile phosphate metabolites. Hence it is concluded that the ^{31}P spectra observed from the tumor bearing foot represent the spectra derived only from the tumor tissues. Anesthesia was not used in our study whereas it is routinely used in most other studies of tumor. Anesthesia causes known and variable effects on blood pressure, body temperature, tumor blood flow, pH and ^{31}P MRS spectrum. Spectra obtained from murine RIF-1 fibrosarcoma when the host animal was under pentobarbital anesthesia showed a decline in high energy phosphates.[21, 22] Due to these difficulties with anesthesia, we chose to use physical restraint to maintain the animal in a stationary position.

Whereas no statistical differences were found in priming (-) group, 31P MRS parameters markedly improved from baseline after test dose of GBE in priming(+) group. The reduction in Pi/ATP and the increase in PCr/Pi, PCr/(Pi+PME) are consistent with the results of our previous studies and suggest that GBE can increase tumor blood flow significantly, in favor of the GBE priming. But changes of pH and PME/ATP in priming(+) group did not show statistical significance. Tumor hypoxia can result in lactate production through glycolysis, with a resulting acidification of tumor pH. Estimation of tumor pH from the chemical shift of the Pi can suffer from a number of potential sources of error, as ionic strength, metal ion binding, and temperature all influence the chemical shift of Pi.[23] In general tumor pH undergoes little change after manipulation in many tumors, suggesting that the inherent buffering capacity of tumor cells also may be important in determining tumor pH.[24] Phosphomonoesters (PME), primarily phosphocholine and phosphoethanolamine are both precursors of phospholipid synthesis and products of membrane catabolism. It has been observed that the intensity of these signals correlated with increasing tumor size suggesting that elevated levels result from breakdown of membrane components in necrotic regions.[15] In the small tumor used in this experiment, PME signal might be influenced little by the blood flow modifiers such as GBE.

Because complete radiobiologic hypoxia occurs at a lower oxygen tension than metabolic hypoxia, we anticipate that any tumor that exhibits a radiobiologic hypoxia will have a metabolic hypoxia also. Thus there have been many attempts to predict radiobiologic hypoxia from ^{31}P MRS spectra.[24~27] But it should be noted that radiobiologic hypoxia and metabolic hypoxia are distinct from each other.[28, 29] Several possible explanations may account for the low correlations between radiobiologic hypoxia and metabolic ratios. First, ^{31}P MRS results are weighted averages of signal from nearly the whole tumor. Hypoxic fraction measurements, on the other hand, are based on variations in survival of very small fraction of clonogenic cells. Secondly, acute changes in tumor oxygenation can occur instantaneously. Thirdly, biological variation, experimental error, S/N ratio, chronic hypoxic area which do not produce high energy metabolite are also contributing factors.[30] Also a difference may exist in how these types of hypoxia are affected by blood flow modifiers. Tumor reoxygenation is generally expected to affect only the radiobiologic hypoxia and to result in those cells becoming more sensitive to radiation. However, radiobiologically hypoxic cells that undergo reoxygenation could still be suffering metabolic hypoxia. Furthermore, metabollically hypoxic cells can undergo reoxygenation, to become fully oxic cells, without necessarily having an effect on radioresistance of the tumor. This suggests that metabolic reoxygenation can be an event distinct from radiobiologic reoxygenation. Therefore, quantitative relationship between the effects on tumor blood perfusion, radiobiologic hypoxic fraction and alterations in parameters of the ^{31}P MRS are unclear at this time. However, preliminary data suggest that ^{31}P MRS may prove useful for determining the effectiveness of therapeutic inter-

ventions designed to manipulate radiobiologic hypoxia in tumors.

In conclusion, despite of above limitations, Ginkgo biloba extract certainly improve energy metabolic status by increasing tumor blood flow and could be used as a potential radiosensitizer for the malignant tumor, at least in this FSaII tumor model. But, to gain the general acceptance of GBE as a radiosensitizer, numerous experiment in other tumor model and clinical trial should be committed.

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국문 초록

Gingko biloba extract가 C3H 마우스 섬유육종의 에너지 대사 상태에 미치는 영향 : 생체내 ^{31}P 자기공명 분광법을 이용한 분석

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목 적 : 현재까지 방사선에 대한 저산소세포의 감수성을 높이기 위한 많은 실험적 및 임상적 연구가 진행되어 왔으나, 아직 적절한 방법이 개발되지 못한 상태이다. 본 연구에서는 혈관수축 이완 및 혈액점도 저하를 통하여 말초혈류 증가작용을 갖고 있는 Ginkgo biloba extract (GBE)가 종양내 대사상태에 어떠한 변화를 가져오는지를 ^{31}P 핵자기 공명 분광법을 통하여 알아보고자 하였다.

방 법 : 100 mm^3에서 130 mm^3의 섬유육종을 갖는 18마리의 C3H 마우스를 각각 9 마리씩 두 군으로 분리하였다. 한 군은 GBE로 전처치를 하지 않았으며 나머지 다른 한 군은 ^{31}P 자기공명분광법을 시행하기 24시간 전에 100 mg/kg의 GBE를 복강내로 투여하여 전처치를 하였다. 우선 각 군에서 ^{31}P 자기공명분광법을 실시하여 대조 spectrum을 얻었으며 그 후 100 mg/kg의 GBE를 재투여 하고 약 1시간 후에 ^{31}P 자기공명분광법을 다시 실시하였다.

결 과 : 전처치를 하지 않은 군에서는 GBE 투여 전의 평균 pH, PCr/Pi, PME/ATP, Pi/ATP, PCr/(Pi+PME) 수치를 GBE 투여 후 1시간에 측정한 값과 비교하였을 때 통계학적인 차이가 없었다. 그러나 전처치를 한 군에서는 GBE 투여 전의 평균 PCr/Pi, Pi/ATP, PCr/(Pi+PME)수치가 0.49, 0.77, 0.17에서 GBE 투여 후에는 0.74, 0.57, 0.28로 변화하였으며 이는 paired-t test상 통계학적으로 의미있는 차이였다.

결 론 : 전처치를 한 군에서 GBE의 재투여로 대사상태가 현저히 호전되었으며 이는 간접적으로 GBE 에 의한 방사선감수성의 증가가 혈류 증가 및 이에 따른 대사상태 호전에 기인함을 나타낸다.

핵심용어 : Ginkgo biloba extract, 마우스 섬유육종, 에너지 대사, ^{31}P 자기공명분광법