Studies on Experimental Approach for Evaluation about Efficacy and Safety of Antioxidants

（抗酸化物質の有効性および安全性に関する実験的研究）

2014

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General Introduction

The vast majority of complex life including animals as well as plants on earth requires oxygen for its existence, but the highly reactive molecule oxygen paradoxically damages living organisms by producing reactive oxygen species [17]. These reactive oxygen species can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins [89]. Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms [55, 108], while damage to proteins causes enzyme inhibition, denaturation, and protein degradation [92]. Thus organisms contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such as DNA, protein and lipids [17, 110].

In general, antioxidant systems either prevent these reactive oxygen species from being formed, or removed them before they can damage vital components of the cell [17, 89]. However, reactive oxygen species also have useful cellular signaling, such as reduction/oxidation (redox). So, it is likely that the function of antioxidant systems is not to remove oxidants entirely, but instead to keep them at an optimum level [80]. Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cells, including proteins, lipids and DNA.

In humans, oxidative stress is thought to be involved in the development of a variety of diseases such as cancer [24], Parkinson's disease, Alzheimer's disease [109], atherosclerosis, heart failure [90], myocardial infarction[77], fragile X syndrome [19], Sickel cell disease [3], lichen planus [2], vitiligo [8], autism [31], and chronic fatigue syndrome [35] although reactive oxygen species can be beneficial, as they are used by the immune system as a way to attack and kill

pathogens [86]. Short-term oxidative stress may also be important in prevention of aging induction of a process named mitohormesis [22].

The use of antioxidants to prevent disease is controversial [53]. In a high-risk group like smokers, high doses of synthetic β-carotene increased the rate of lung cancer [81]. In less high-risk groups, the use of vitamin E appears to reduce the risk of heart disease, although more recent evidence may in fact suggest the opposite [73]. In other diseases, such as Alzheimer's disease, the evidence on vitamin E supplementation is mixed [12, 45]. Since dietary sources contain a wider range of carotenoids and vitamin E (tocopherols and tocotrienols) from whole foods, ex post facto epidemiological studies can have differing conclusions than artificial experiments using isolated compounds.

Although oxidation reactions are crucial for life, they can also be damaging; plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Insufficient levels of antioxidants, or inhibition of antioxidant enzymes, cause oxidative stress and may damage or kill cells. Antioxidants are widely used in dietary supplements and have been investigated for the prevention of diseases such as cancer, coronary heart disease and even altitude sickness. Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials with a limited number of antioxidants detected no benefit and even suggested that excess supplementation with certain putative antioxidants may be harmful [9, 11, 32]. To further clarify such controversial evidence experimentally, representative antioxidants were assessed for the risk and benefit.

In the present studies, I evaluated the biological responses by administration of three kinds of antioxidants to some designed studies. Tocotrienol are members of the vitamin E family. Vitamin E is an essential nutrient and is used as food ingredient and food supplement. Tocotrienols

possess excellent antioxidant activity in vitro and have been suggested to suppress reactive oxygen species production more efficiently than tocopherols [84]. In the chapter 1, tocotrienols was administered to rat orally for 13weeks and evaluated toxicological profiles of the substance by referring to OECD repeated dose toxicity protocol.

Protocatechuic acid is a dihydroxybenzoic acid and is in edible plants (mushroom, roselle) [18, 50], fruits (Açaí palm) [66], and vegetables. This substance has also antioxidative property, and is known to exert chemopreventive activity on tumorigenesis in various organs on rodent cancer model studies. In the chapter 2, modifying effects of protocatechuic acid to proliferative lesions were evaluated by using BOP-initiated Hamster model for pancreatic cancer.

Oltipraz is antischistosomal agent. This substance act by reducing glutathione (GSH) stores of parasites, but also increases level of GSH in many tissue of the host [67]. Oltipraz has antioxidative activity and show chemopreventive action in many in vitro/in vivo studies [16, 37, 39, 65]. It was reported that Phase II trials of oltipraz for therapy in liver fibrosis or cirrhosis [41]. In the chapter 3, modifying effects of oltipraz to liver defects were evaluated by administration to LEC rat which accumulated Cu in liver and developed liver lesions.

Chapter 1

Oral toxicity of a tocotrienol preparation in rats

1-1 Introduction

Tocotrienols are minor plant constituents, especially abundant in cereal grains, palm oil and in latex, which can provide a significant source of vitamin E-like activity. They possess general structural features of vitamin E: an aromatic chromanol head and a 16-carbon hydrocarbon tail, with differences in the methyl substituent in the chromanol nucleus giving rise to α-, β-, γ- and δ-isomers (Figure. 1). Tocotrienols have antioxidant properties like tocopherols [87] , and are used as food additives to prevent oxidization. Various antioxidants such as alpha-tocopherol, and including preparations containing tocotrienols have been shown to have chemopreventive potential against cancer [23, 44, 94]. Moreover, tocotrienols manifest intrinsic hypocholesterolemic activity in vivo or in vitro, which has not been reported for tocopherols [67, 68, 74, 75, 76, 112]. Elevated serum cholesterol is a major risk factor for atherosclerosis and use of tocotrienols as a functional food is therefore possible. However, there have been not enough reports of toxicological evaluation of tocotrienols. Therefore, we performed a 13-week oral toxicological study of tocotrienols in rats for safety assessment.

1-2 Materials and Methods

Experimental animals and housing conditions

Specific pathogen-free F344/DuCrj male and female rats (5 weeks old) were purchased from Charles River Japan (Atsugi, Japan) and acclimated for 1 week prior to commencement of the test. Rats were randomly allocated to five groups, each consisting of 10 males and 10 females, and housed in a room with a barrier system, maintained under the following conditions: temperature of 24±1℃, relative humidity 55±5%, ventilation frequency of 18 times/h and a 12-h light cycle. The animals were housed in plastic cages (five rats/cage) on chip bedding, and given tap water ad lib.

Chemicals and administration

The tocotrienol preparation (Tama Biochemistry Co., Ltd, Tokyo, Japan) was kindly provided by Eisai Co., Ltd (Tokyo) as a yellow–brown viscous liquid. It was purified from a vitamin E mixture obtained from palm oil by molecular distillation. Complete dissociation from tocopherol was impossible and the mixture used in this study had the following composition: α-tocotrienol 21.4%, β-tocotrienol 3.5%, γ-tocotrienol 36.5%, δ-tocotrienol 8.6%, α-tocopherol 20.5%, β-tocopherol 0.7%, γ-tocopherol 1.0% and δ-tocopherol 0.5%.

Groups of 10 rats of each sex were fed diet containing 0 (group 1), 0.19 (group 2), 0.75 (group 3) and 3% (group 4) tocotrienol preparation ad lib. for 13 weeks. The high-dose level of 3% was determined based on a pilot study in which 5% but not 3% tocotrienol diet was refused by rats. CRF-1 powder diet (Oriental Yeast Co., Ltd, Tokyo) was used as a basal diet. Diets were prepared every 7 days based on stability data, with no significant reduction in tocotrienol levels being noted in the remaining diet even after 7 days. Because the chemical used in this study contained tocopherol, another group (group 5) was fed diet containing 0.69% alpha-tocopherol (Tama Biochemistry Co., Ltd), which was identical to the concentration of tocopherol in the high-dose (3%) group, to assess

the influence of tocopherol itself.

Observation and examination methods

Clinical signs were observed once a day. Body weights and food intake were measured once a week. Hematological and serum biochemical examinations as well as necropsy were performed at the end of the experiment, when blood samples were collected from the abdominal aorta under ether anesthesia after 16 h of starvation.

Hematological parameters, such as the red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), white blood cell count (WBC) and white blood cell differential count, were assessed with an automated hematology analyzer (Sysmex CC-150, TOA Medical Electronics Co., Ltd, Hyogo, Japan).

Serum biochemical assessments were conducted for 17 items; total protein (TP), albumin/globulin ratio (A/G), albumin (AlB), total bilirubin (T.Bil), triglyceride (TG), total cholesterol (T.Chol), blood urea nitrogen (BUN), creatinine (CRN), asparagine transaminase (AsT), alanine transaminase (AlT), γ-glutamyl transaminase (γ-GT) and alkaline phosphatase (ALP) using a Gemsaec Auto Analyzer Type 4 (Electro Nucleonics, USA) and calcium (Ca), inorganic phosphate (P), sodium (Na), potassium (K) and chloride (Cl) using a Hitachi Electrolyte Analyzer 702 (Hitachi Ltd, Tokyo, Japan).

At autopsy, weights of brain, heart, lungs, thymus, liver, spleen, adrenals, kidneys, testes, ovaries and uterus were measured. The above-mentioned organs and the nasal cavity, pituitary, eyeballs, Harderian glands, spinal cord, salivary glands, stomach, small and large intestine, pancreas, urinary bladder, skin, mammary gland, mesenteric lymph nodes, trachea, esophagus, thyroid gland, tongue, skeletal muscle, ischiatic nerve, epididymis, seminal vesicles, prostate gland, vagina and

bone marrow (femur and sternum) were fixed in 10% buffered formalin solution for routine histopathological processing. Paraffin sections were stained with hematoxylin and eosin. Histopathological examinations were carried out on groups 1 and 4 for both sexes. When lesions were only observed in group 4, the affected organs in other groups were also examined.

Statistical analysis

The data for body and organ weights, food consumption, hematology and serum biochemistry were analyzed statistically as follows. The Bartlett's test was applied to test homogeneity of variance between groups. If no significant heterogeneity was detected, one-way analysis of variance was applied. The parameters found to be significant in the one-way analysis of variance were tested by Dunnett's multiple comparison. If significant heterogeneity of variance was detected, the Kruskal–Wallis test was conducted. The parameters found to be significant in the Kruskal–Wallis test were tested by the non-parametric equivalent of Dunnett's or Scheffe's multiple comparisons. The data for lesion incidences were analyzed using the Fisher's exact test. P values less than 0.05 were considered significant.

1-3 Results

General signs

No deaths occurred and no remarkable changes in general appearance were observed.

Body weight and food intake

During treatment, suppression of body weight gain was observed in group 4 males (Figure. 2). Data for food and tocotrienol intake are shown in Table 1. Food intake was comparable among the groups throughout the administration period for both sexes so that actual tocotrienol intake was close to the nominal values.

Hematological and serum biochemical data

The results of hematological and serum biochemical examinations are summarized in Tables 2 and 3. With hematology, significant decreases of MCV were observed in all treated males and PLT was significantly decreased in group 3 and 4 males. A significant decrease in MCH and a significant increase in WBC were also observed in group 4 males. Hb, MCV, MCH and MCHC were significantly decreased in group 3 and 4 females along with Ht in the latter group. In white blood cell differential counts, a significant decrease in monocytes was observed in group 4 males. Band form leukocytes were significantly decreased in group 3 males, but no dose relation was apparent. Significant decreases in segmented leukocytes and monocytes, and significant increases in lymphocyte were observed in group 3 and 4 females. Eosinophilic leukocytes were significantly decreased in group 4 females.

Regarding serum biochemistry, A/G and ALP were significantly increased in all treated males. T.Cho was significantly decreased in group 3 and 4 males. Significant increases in AlB, BUN, Ca and AlT, and a significant decrease in P, were observed in group 4 males. Na was

significantly decreased in group 3 males, but without any dose relation. A significant decrease in Na and increase in K were observed in group 3 and 4 females. A/G, TG, BUN, P, AlT, γ-GT and ALP were significantly decreased in group 4 females. AsT was significantly increased in group 4 and significantly decreased in group 2 females. Ca was significantly increased in group 2 females, but without any dose relation. In group 5 males, significant decreases in TG and CRN, and significant increases in AsT, AlT and ALP were observed. Significant decreases in BUN and AsT, and a significant increase in Ca were also noted in group 5 females. γ-GT values in both sexes were below the detection limit except in group 4 females.

Organ weights

The relative organ weights are shown in Table 4. Significant increases in adrenal weights were observed in all treated males. Brain, heart, liver, kidneys and testes weights were also significantly increased in group 4 males. Significant decreases and a tendency for reduction of lung weights were observed in all treated females. Significant increases in liver and spleen weights, and significant decreases in ovaries and uterus weights were observed in group 4 females. A significant elevation in the liver weight was observed in group 5 females.

Histopathological examination

The results of histopathological examination are summarized in Table 5. Slight hepatocellular hypertrophy in group 3 and 4 males and reduction of cytoplasmic vacuolation in adrenal cortical region in group 4 males were observed. There were no other lesions attributable to the tocotrienol treatment in either sex.

1-4 Discussion

In the present 13-week toxicity study of tocotrienols, suppression of body weight gain was observed as a major clinical change in males given 3% tocotrienol. However, food intake mirrored that in the basal diet group throughout the administration period so that the cause of the decrease in body weight was unclear.

On hematological examination, decreases in MCV and MCH were noted in males but there was no change of RBC, Hb or Ht, and neither of these appeared to be of toxicological significance, since the degrees were small and no related histopathological changes were found. In females, changes of parameters related to anemia (decreases in Hb, Ht, MCV, MCH, and MCHC) with significant increase in spleen weights were observed. However, as in males, the RBC did not differ between the treated and the control groups, and again no histopathological changes related to these parameters were apparent. In males, an increase in WBC and a reduction of monocytes were observed and in females, decreases in segmented leukocytes and eosinophilic leukocytes and monocytes, and an increase in lymphocytes were noted. However, the degrees of changes were small and histopathological examination did not reveal any evidence in the femur or sternum. Therefore, the findings are probably of little toxicological significance. In contrast, PLT was decreased in males although not females in a dose-dependent manner, pointing to a physiological response. Although no hemorrhagic lesions were found in the present study, a decrease in PLT may be related to the hemorrhagic toxicity of large doses of tocopherols [102]. In this context, it is interesting that α-tocopherol inhibits aggregation of human platelets by a protein kinase C-dependent mechanism [20].

On serum biochemical examination, a decrease in total cholesterol was observed in males, in line with the known hypocholestrolemic activity of tocotrienols in vitro or in vivo through inhibition of 3-hydroxy-3- methylglutaryl-coenzyme A (HMG-CoA) reductase, a rate-limiting

enzyme of cholesterol synthesis tocopherols [67, 68, 74, 75, 76, 112]. An increase in AlT was observed in both sexes, as well as elevated AsT and γ-GT in females. Moreover, liver weight increase was observed in both sexes, with slight hepatocellular hypertrophy in males. In fact, AsT and AlT are known to be induced in rats treated with various HMG-CoA reductase inhibitors [1, 71], and changes in liver may result from inhibition of the key enzyme of the mevalonate pathway. However, similar changes in AsT and AlT were also observed in tocopherol-treated males, so that the possibility that tocopherol in the preparation had a certain influence must be borne in mind. Increases in ALP, observed in both sexes, may be caused by cholestasis or bone remodeling, but there was no increase of total bilirubin or any histological evidence of cholestasis in this study. While Ca and P, bone-remodeling related parameters, were changed in both sexes, the changes were small and not dose dependent without any associated histological correlate. Thus the results indicate that ALP increase was caused by the treatment, but this was not of major toxicological importance. Increases in BUN were observed in both sexes, and decreased Na and increased K were noted in females, but there was no increase in creatinine, and no lesions were apparent in the kidneys on histopathological examination. Increases in A/G were observed in both sexes and an increase in albumin in males, but the degrees of change were small, and related toxicological changes (such as dehydration and thyroid function insufficiency) were not evident. Thus the findings for all these parameters may be of little toxicological significance.

Relative weights of the adrenals were dose-dependently increased in all treated males. In addition, reduction of vacuolation in the adrenal cortical region was observed histopathologically. It is known that the relevant cells contain large cytoplasmic lipid droplets which consist of cholesterol and other steroid precursors, so that the region may be similar to the cytoplasmic lipid droplets, and the reduction of vacuolation could have been caused by hypocholestrolemic activity of tocotrienols. In females, reduction of the ovary and uterus weights was observed. Although there

were no accompanying histopathological changes, the possibility that there was a certain influence of reduction in cholesterol on the production of sex hormones must be considered, since reproductive organ weights are highly sensitive to variation in sex hormone levels. Lung weights were reduced or showed a tendency for reduction in all dose groups, but the degrees of changes were small and again there were no histopathological changes. The increases in brain, heart, kidneys and testes weights observed in the 3% males were possibly due to suppression of body weight gain.

In this study, because of the histological changes in male livers and hematological changes in females, the NOAEL was concluded to be 0.19% in the diet (120 mg/kg body weight/day for male rats and 130 mg/kg body weight/day for female rats). As the decrease in MCV, increase in A/G and ALP, and elevation of adrenal weights were observed in all treated males, a NOEL could not be determined.

1-5 Summary

Tocotrienols are added as antioxidants to food. As there have been no reports of toxicological evaluation, a 13-week oral toxicity study was performed in Fischer 344 rats of both sexes at dose levels of 0 (group 1), 0.19 (group 2), 0.75 (group 3) and 3% (group 4) of a preparation in powdered diet. Suppression of body weight gain was observed in group 4 males. On hematological examination, significant decrease in mean corpuscular volume (MCV) was observed in all treated males. Platelets were significantly reduced in group 3 and 4 males. Hemoglobin concentration, MCV, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were significantly decreased in group 3 and 4 females and hematocrit in group 4 females. On serum biochemical examination, increase in the albumin/globulin ratio (A/G) and alkaline phosphatase in all treated males, elevated alanine transaminase in group 4 of both sexes and increases in asparagine transaminase and γ-glutamyl transaminase in group 4 females were observed.

With regard to relative organ weights, liver weights in group 4 of both sexes and adrenal weights in all treated males demonstrated an increase, and ovary and uterus weights in group 4 females were reduced. Histopathologically, slight hepatocellular hypertrophy in group 3 and 4 males, and reduction of cytoplasmic vacuolation in the adrenal cortical region in group 4 males were observed.

Because of pathological changes in male liver and hematological changes in females, the no-observed-adverse-effect level (NOAEL) was concluded to be 0.19% in the diet (120 mg/kg body weight/day for male rats and 130 mg/kg body weight/day for female rats).

As a decrease in MCV, an increase in the A/G, elevation of alkaline phosphatase and increase in adrenal weight were observed in all treated males, a no-observed-effect level (NOEL) could not be determined in this examination.

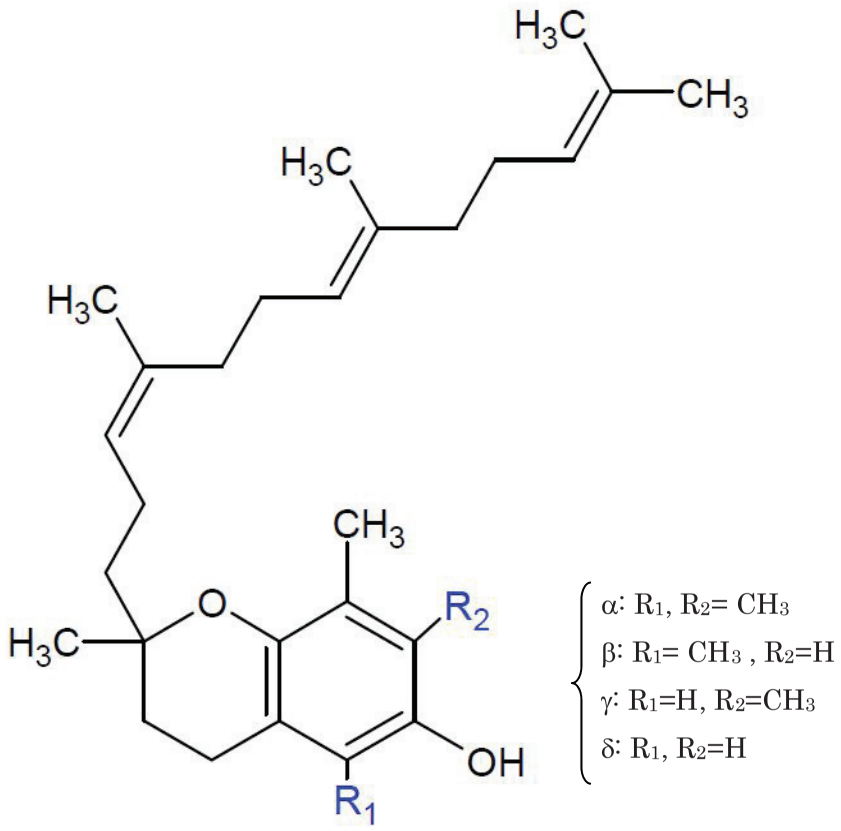

Figure 1. Chemical structures of tocotrienols.

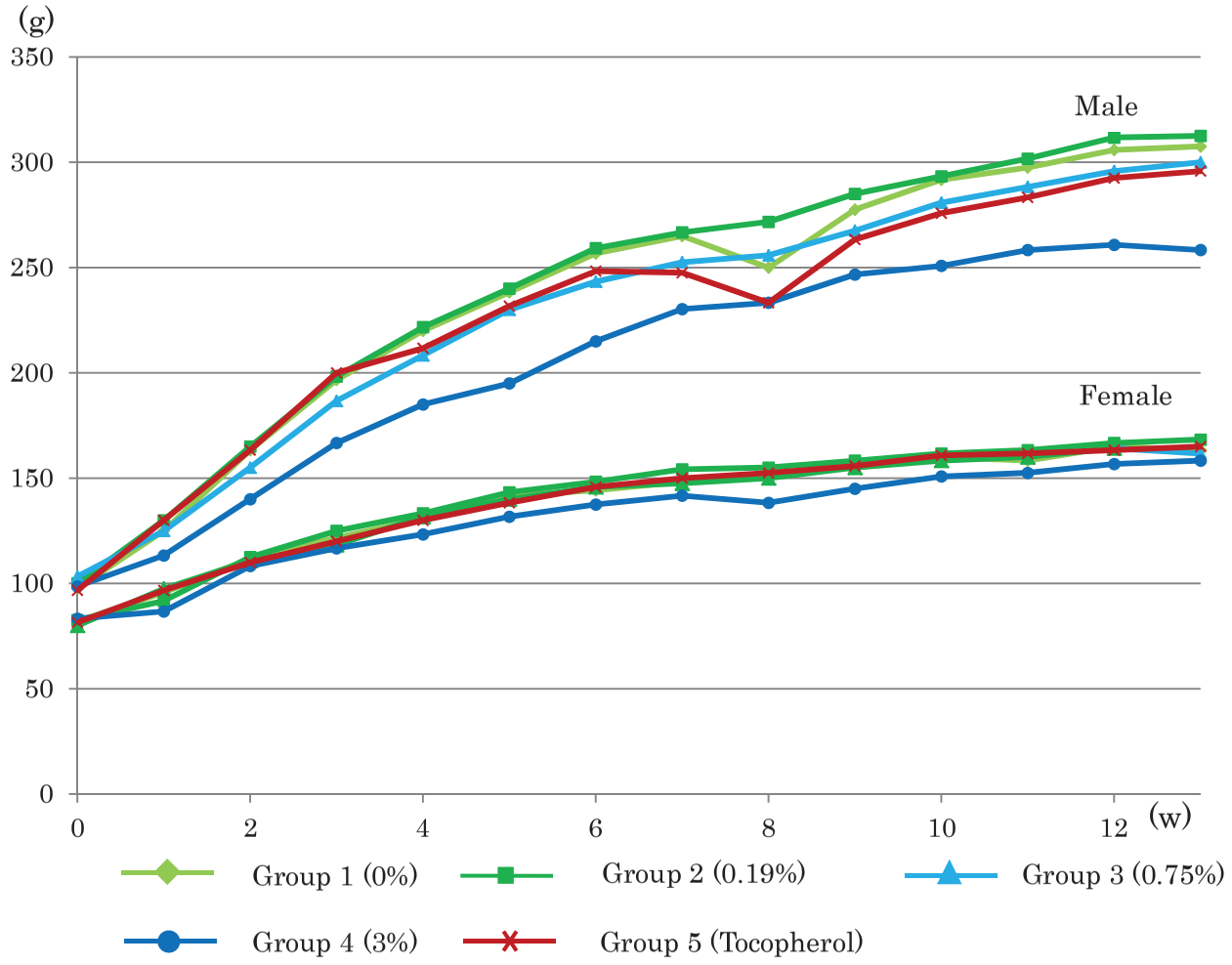

Figure 2. Body weight curves of rats

Table 1. Food consumption and intake of tocotrienol

Dose level	Food consumption (g/animal/day)		Daily intake (mg/kg/day)		Total intake (g/kg)	
	Male	Female	Male	Female	Male	Female
Group 1 (0%)	14.2	9.4	-	-	-	-
Group 2 (0.19%)	14.7	9.4	119.0	129.8	10.7	11.7
Group 3 (0.75%)	14.2	9.1	474.2	491.0	42.7	44.2
Group 4 (3%)	14.4	9.0	2130.3	2046.6	191.7	184.2

Table 2. Hematological and serum biochemical data of F344 male rats treated with tocotrienol for 13 weeks

Item	Tocotrienol				Tocopherol
	Group 1 (0%)	Group 2 (0.19%)	Group 3 (0.75%)	Group 4 (3%)	Group 5 (0.69%)
RBC (10^4/mL)	920.3±35.0[a]	920.3±35.0	920.3±35.0	920.3±35.0	920.3±35.0
Hb (g/dL)	15.2±0.5	15.0±0.3	14.7±0.5	14.8±1.0	15.3±0.4
Ht (%)	44.3±1.7	43.4±1.1	42.5±1.4	43.3±3.0	44.6±1.0
MCV (FL)	48.1±0.3	47.6±0.8[c]	47.1±0.3[d]	47.2±0.5[d]	48.2±0.4
MCH (pg)	16.5±0.4	16.4±0.2	16.3±0.1	16.1±0.1[d]	16.5±0.2
MCHC (g/dL)	34.3±0.9	34.6±0.3	34.6±0.4	34.1±0.3	34.3±0.3
PLT (10^4/mL)	76.8±3.7	75.6±4.0	71.3±3.6[c]	68.7±5.6[d]	74.4±5.1
WBC (10^2/mL)	36.1±6.7	42.2±4.7	36.5±4.4	44.0±9.8[c]	40.6±6.8
Band [b] (%)	0.3±0.4	0.1±0.2	0	0.1±0.2	0.3±0.3
Seg (%)	19.3±3.3	21.5±2.8	21.4±4.1	21.3±5.4	22.2±4.2
Eosi (%)	1.1±0.7	0.9±0.7	0.9±1.0	1.3±0.9	1.3±0.7
Baso (%)	0	0	0	0	0
Lymp (%)	77.7±3.5	76.0±3.6	76.4±3.5	76.8±6.1	74.9±4.2
Mono (%)	1.7±0.9	1.5±0.8	1.2±0.8	0.4±0.6[d]	1.4±1.0
TP (g/dL)	6.0±0.3	6.0±0.3	5.8±0.3	6.0±0.1	6.0±0.2
A/G	2.5±0.2	2.7±0.2[c]	2.9±0.2[c]	3.5±0.3[d]	2.7±0.2
AlB (g/dL)	4.3±0.2	4.4±0.2	4.3±0.2	4.7±0.1[c]	4.4±0.1
T. Bil (mg/dL)	0.19±0.28	0.10±0.00	0.10±0.00	0.11±0.03	0.10±0.00
TG (mg/L)	44.2±11.1	56.3±14.6	45.7±16.1	55.9±10.9	38.6±12.9
T. Cho (mg/dL)	65.3±8.1	61.5±3.1	53.3±4.0[d]	53.7±5.9[d]	59.5±7.8
BUN (mg/L)	16.0±1.9	17.9±1.7	18.9±1.7	22.0±4.1[d]	16.4±1.1
CRN (mg/dL)	0.27±0.05	0.26±0.05	0.26±0.05	0.30±0.00	0.22±0.04[c]
Ca (mg/dL)	9.6±0.4	10.0±0.3	9.6±0.3	10.1±0.5[c]	9.9±0.2
P (mg/dL)	5.8±0.4	6.0±0.3	5.4±0.4	5.0±1.1[c]	5.8±0.2
Na (mEQ/L)	142.5±1.4	142.2±0.6	141.2±1.1[c]	142.3±1.4	142.3±0.9
K (mEQ/L)	4.5±0.2	4.3±0.3	4.4±0.4	4.6±0.4	4.4±0.3
Cl (mEQ/L)	103.3±1.3	103.8±0.8	104.2±0.6	104.5±2.1	104.6±1.3
AsT (IU/L)	79.1±9.2	77.5±13.8	86.1±17.3	90.6±6.2[d]	153.4±89.6[d]
AlT (IU/L)	50.7±6.0	54.7±6.6	50.9±5.6	78.4±9.3	103.8±51.7[d]
AlP(IU/L)	326.5±21.4	365.8±23.9	393.7±44.3[d]	437.6±38.4[d]	366.5±32.3[c]

[a] Mean ± S.D.

[b] Band; Band form leukocyte, Seg; Segment leukocyte, Eosi; Eosinophilic leukocyte, Baso; Basophilic leukocyte, Lymp; Lymphocyte, Mono; Monocyte

[c] Significantly different from the control group at $P<0.05$.

[d] Significantly different from the control group at $P<0.01$.

Table 3. Hematological and serum biochemical data of F344 female rats treated with tocotrienol for 13 weeks

Item	Tocotrienol				Tocopherol
	Group 1 (0%)	Group 2 (0.19%)	Group 3 (0.75%)	Group 4 (3%)	Group 5 (0.69%)
RBC (10^4/mL)	870.7±37.0[a]	862.4±27.7	859.7±23.1	852.4±30.7	891.0±30.2
Hb (g/dL)	16.0±0.6	15.6±0.5	15.2±0.4[d]	14.7±0.4[d]	16.3±0.5
Ht (%)	43.6±1.7	43.2±1.3	42.7±1.1	41.9±1.4[c]	44.5±1.5
MCV (FL)	50.1±0.3	50.1±0.2	49.6±0.2[d]	49.2±0.5[d]	50.0±0.5
MCH (pg)	18.3±0.2	18.1±0.2	17.7±0.1[d]	17.2±0.3[d]	18.2±0.2
MCHC (g/dL)	36.6±0.4	36.2±0.4	35.7±0.3[d]	35.0±0.5[d]	36.5±0.3
PLT (10^4/mL)	75.9±7.4	79.1±5.2	78.0±2.5	74.3±5.0	77.5±3.1
WBC (10^2/mL)	32.8±4.1	33.0±3.7	28.3±5.1	37.5±5.1	33.7±6.8
Band [b] (%)	0.1±0.2	0.1±0.2	0.1±0.2	0.1±0.2	0.1±0.3
Seg (%)	20.4±3.4	21.2±2.8	16.0±2.5[d]	13.2±2.0[d]	17.5±4.7
Eosi (%)	1.1±0.7	0.8±0.5	0.9±0.2	0.5±0.6[c]	0.6±0.6
Baso (%)	0	0	0	0	0
Lymp (%)	76.3±3.4	76.7±3.3	82.3±2.6[d]	85.8±2.4[d]	79.9±5.1
Mono (%)	2.1±1.2	1.3±0.9	0.7±0.9[d]	0.6±0.6[d]	2.0±1.1
TP (g/dL)	5.9±0.2	5.9±0.2	5.8±0.2	5.8±0.1	6.1±0.2
A/G	3.2±0.2	3.1±0.3	3.3±0.2	3.7±0.2[d]	3.1±0.2
AlB (g/dL)	4.5±0.1	4.5±0.1	4.5±0.1	4.6±0.1	4.6±0.2
T. Bil (mg/dL)	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.00
TG (mg/L)	13.4±2.41	17.5±4.6	14.8±2.5	29.1±6.0[d]	12.8±3.9
T. Cho (mg/dL)	82.0±7.6	87.6±7.9	86.63±7.9	75.0±6.1	86.6±10.3
BUN (mg/L)	15.7±1.1	16.1±1.3	16.5±0.6	20.1±1.8[d]	17.9±2.8[c]
CRN (mg/dL)	0.29±0.03	0.25±0.05	0.29±0.03	0.27±0.05	0.30±0.00
Ca (mg/dL)	9.6±0.2	9.8±0.2[d]	9.7±0.1	9.7±0.1	9.9±0.4[d]
P (mg/dL)	5.4±0.4	5.6±0.2	5.4±0.4	5.8±0.4[c]	5.6±0.7
Na (mEQ/L)	144.5±1.0	143.9±0.9	143.4±0.8[c]	142.2±1.0[d]	144.2±0.8
K (mEQ/L)	3.7±0.2	3.9±0.3	4.1±0.2[d]	4.5±0.2[d]	3.8±0.3
Cl (mEQ/L)	106.8±1.1	107.6±1.0	107.4±0.8	107.0±0.8	106.6±2.8
AsT (IU/L)	80.8±6.7	70.3±3.2[d]	78.0±5.2	97.76±8.4[d]	71.6±5.1[d]
AlT (IU/L)	39.3±3.6	41.57±5.0	37.1±2.3	58.4±10.0[d]	42.8±7.1
AlP(IU/L)	256.4±29.5	276.0±33.2	278.7±22.2	413.5±31.9[d]	282.0±34.1

[a] Mean ± S.D.

[b] Band; Band form leukocyte, Seg; Segment leukocyte, Eosi; Eosinophilic leukocyte, Baso; Basophilic leukocyte, Lymp; Lymphocyte, Mono; Monocyte

[c] Significantly different from the control group at $P<0.05$.

[d] Significantly different from the control group at $P<0.01$.

Table 4. Relative organ weights of rats treated with tocotrienol for 13 weeks

	Tocotrienol				Tocopherol
	Group 1 (0%)	Group 2 (0.19%)	Group 3 (0.75%)	Group 4 (3%)	Group 5 (0.69%)
Male					
Body weight (g)	290.9±18.9[a]	296.4±10.2	281.8±21.1	244.9±14.5[c]	276.7±14.7
Brain (g%)	0.66±0.04	0.65±0.02	0.68±0.04	0.77±0.04[c]	0.68±0.04
Heart (g%)	0.34±0.10	0.30±0.01	0.30±0.01	0.32±0.01	0.30±0.01
Thymus (g%)	0.065±0.011	0.062±0.005	0.058±0.010	0.056±0.006	0.058±0.008
Lungs (g%)	0.331±0.015	0.319±0.025	0.318±0.017	0.349±0.016	0.312±0.013
Liver (g%)	2.28±0.11	2.34±0.05	2.31±0.06	2.60±0.10[c]	2.30±0.10
Spleen (g%)	0.20±0.01	0.19±0.01	0.19±0.01	0.21±0.02	0.19±0.01
Adrenals (g%)	0.011±0.001	0.013±0.001[b]	0.013±0.002[b]	0.016±0.002[c]	0.012±0.002
Kidneys (g%)	0.63±0.03	0.63±0.02	0.65±0.03	0.69±0.03[c]	0.65±0.02
Testes (g%)	1.05±0.05	1.04±0.03	1.04±0.03	1.14±0.06[c]	1.10±0.07
Female					
Body weight (g)	149.8±8.8	154.2±8.1	152.6±8.9	145.3±6.7	164.5±5.6
Brain (g%)	1.17±0.06	1.12±0.08	1.15±0.07	1.18±0.05	1.13±0.07
Heart (g%)	0.36±0.02	0.37±0.06	0.35±0.02	0.35±0.02	0.36±0.03
Thymus (g%)	0.104±0.012	0.107±0.009	0.098±0.012	0.100±0.008	0.107±0.008
Lungs (g%)	0.48±0.05	0.43±0.03[b]	0.43±0.3	0.43±0.05[b]	0.43±0.03
Liver (g%)	2.27±0.09	2.20±0.07	2.22±0.05	2.44±0.10[c]	2.47±0.14[c]
Spleen (g%)	0.23±0.01	0.22±0.01	0.23±0.01	0.25±0.02[c]	0.23±0.02
Adrenals (g%)	0.025±0.004	0.024±0.004	0.024±0.002	0.022±0.004	0.025±0.003
Kidneys (g%)	0.67±0.03	0.68±0.02	0.69±0.03	0.70±0.03	0.68±0.03
Ovaries (g%)	0.039±0.004	0.039±0.003	0.040±0.005	0.033±0.003[c]	0.037±0.005
Uterus (g%)	0.35±0.14	0.35±0.10	0.32±0.09	0.20±0.09[c]	0.31±0.09

[a] Mean ± S.D.

[b] Significantly different from the control group at $P<0.05$.

[c] Significantly different from the control group at $P<0.01$.

Table 5. Histopathological changes in male rats treated with the tocotrienol preparation for 13weeks

		Liver		Adrenals	
		Enlargement of hepatocytes		Reduction of cytoplasmic vacuolation in the cortical region	
	No. of animals	−[a]	+	−[b]	+
Group 1 (0%)	10	10	0	10	0
Group 2 (0.19%)	10	10	0	10	0
Group 3 (0.75%)	10	5	5[c]	10	0
Group 4 (3%)	10	3	7[d]	0	10[d]

[a] −, absent; +, slight.

[b] −, absent; +, present.

[c] $P<0.05$ vs the 0% group values.

[d] $P<0.01$ vs the 0% group values.

Chapter 2

Inhibitory effects of protocatechuic acid on the post-initiation phase of hamster pancreatic carcinogenesis induced by N-nitrosobis(2-oxopropyl)amine

2-1 Introduction

Adenocarcinoma of the pancreatic ducts is the most common pancreatic exocrine malignancy in man. Development of the disease is in general clinically silent so that at the time of diagnosis, the vast majority of cases are incurable, with a very poor prognosis. Epidemiological investigations have indicated that the diet may play an important role in promoting the development of pancreatic cancer, but risk is also reduced by consumption of some dietary factors such as vegetables and fruits [33].

A simple phenolic acid, protocatechuic acid (PCA), a constituent of edible plants, fruits and vegetables [91], is known to exert chemopreventive activity on liver, colon, oral cavity, stomach, urinary bladder and skin carcinogenesis in rodents during the initiation and/or post-initiation phase [25, 64, 96, 97, 98, 99, 105]. It has been postulated that natural phenolic compounds exhibit chemopreventive effects through antioxidant activity or modification of drug metabolizing enzymes in the initiation period and through suppression of cell proliferation in the post-initiation phase [27, 51, 56, 69].

N-Nitrosobis(2-oxopropyl)amine (BOP) has been shown to induce lung, pancreatic, liver and kidney tumors in Syrian hamsters [72]. And BOP-pancreas carcinogenesis model in this animal species has been extensively studied because of the histological and biological similarities between human and hamster pancreatic cancers [72]. Previously, we have shown that several kinds of agents including prostaglandin synthesis inhibitors, trypsin inhibitors and arylalkyl isothiocyanates can prevent pancreatic tumor development induced by BOP [21, 57, 101]. The present study was designed to investigate the inhibitory effects of PCA on pancreatic carcinogenesis induced by BOP when applied in the post-initiation phase.

2-2 Materials and Methods

Animals and chemicals

A total of 120 female Syrian hamsters (Japan SLC, INC., Shizuoka, Japan), 5 weeks old with initial body weights of approximately 80 g, were used in this experiment. The animals were housed five per polycarbonate cage, in an air-conditioned room at 23±2℃ and 60±5% humidity under a daily cycle of alternating 12h periods of light and darkness. Oriental MF (Oriental Yeast, Tokyo, Japan) was freely available as basal diet. BOP was obtained from Nacalai Tesque (Kyoto, Japan) and PCA (97% purity: illustrated in Figure 3) was purchased from Aldrich Chem. (Milwaukee, WI, USA).

Experimental protocol

As schematically illustrated in Figure 4, group 1-6, each consisting of 30 hamsters, were twice given subcutaneous injections of BOP at a dose of 20 mg/kg body weight with a one week interval as the initiation treatment. Starting 1 week after the BOP administration, the animals in groups 1 and 2 were fed a diet supplemented with PCA at doses of 1000 ppm and 500 ppm, respectively, for 49 weeks without prior carcinogen administration.

The hamsters were observed daily and weighed once every 4 weeks. At the end of week 52, all surviving animals were sacrificed and examined. Animals found moribund or dead during the study were also completely autopsied for histological examination. At autopsy, the main target organs for BOP-tumorigenicity including the pancreas, lung, liver and kidney, were carefully examined macroscopically and then fixed in 10% phosphate-buffered formalin. These organs were processed for histological observation by conventional methods, with sections stained with

hematoxylin and eosin. All proliferative lesions were diagnosed histopathologically and counted in representative sections.

The incidence of proliferative lesions was statistically analyzed by the Fisher's exact probability test and the multiplicities of histopathologicaly- defined categories were evaluated by analysis of variance with the Dunnett's T-test. The incidence of macroscopic pancreatic tumors was statistically analyzed by the Mann-Whitney's U-test.

2-3 Results

No clear differences in body weight gain were found and the final body weights were not significantly different among the groups. No significant variation in relative organ weights was apparent and the mortality rates were similar (data not shown). Apart from 3 animals in group 3 which were found dead in an autolytic condition during the early stages of the experiment, all the hamsters were included in the effective numbers.

The data for sizes of macroscopic pancreatic tumors found in BOP-exposed hamsters are shown in Table 6. Pancreatic tumors developed at incidences of 73, 77, and 74% in groups 1, 2 and 3, respectively, with averages of 1.2, 1.1 and 1.1 tumor/hamster, and the differences not being statistically significant. However, the incidence of large sized tumors (>3.0 cm in diameter) was 0, 6 and 23% in groups 1, 2 and 3, respectively; (p<0.05 between group 1 and 3).

Pancreatic proliferative lesions, histopathologically classified as adenocarcinomas and dysplasias, were only noted in the BOP-treated groups with no significant variation in incidence (Figure 5). Multiplicities of adenocarcinomas and dysplasias were also not significantly different among the BOP-treated groups (Figure 6). However, the incidences of advanced pancreatic cancers which had directly invaded adjacent tissues such as the diaphragm, spleen, liver and stomach were decreased by the PCA treatments, being significantly (p<0.01) lower in group 2 than in group 3 (Figure 7).

In other target organs of BOP such as the liver, lung and kidney, neoplastic lesions were induced, but there were no intergroup significant differences (Table 7).

2-4 Discussion

The results of the present study clearly indicated that dietary PCA administration during the post-initiation phase effectively suppressed pancreatic carcinogenesis initiated with BOP as revealed by reduced size of tumors and decreased incidence of invasive cancers. Comparison with earlier results suggests that dietary PCA may be more effective in the late promotion or progression stages than in the initiation or early promotion phase (25, 96, 98, 99).

PCA has been reported to exert chemopreventive activity in various chemically-induced carcinogenesis models using rodents (25, 64, 96, 97, 98, 99, 105). It may inhibit monooxygenase (10), suppress cell proliferation, reduce ornithine decarboxylase (ODC) biosynthesis (25, 64, 97, 98, 99, 105) and scavenge radicals (106, 107, 116). One of the mechanisms underlying the chemopreventive action of PCA may thus be reduction of cell proliferation. In a colon carcinogenesis model in rats, dietary PCA administration at 500 ppm and 1000 ppm significantly reduced the BrdU-labeling index and number of AgNORs, as well as the incidence and multiplicity of colon tumors (97). A similar decrease in cell proliferation with PCA was also found in other studies (25, 64, 98, 99). The fact, found in the present study, that the incidence of large-sized pancreatic tumors was reduced by PCA suggests that suppression of tumor cell division particularly in the progression stage may also be mechanistically involved in the present model.

Many phenolic compounds possess antioxidant or reactive oxygen scavenging activity. PCA, extracted from the rind of Citrus reticulate Blanco, has a 10-times higher antioxidant activity than that of DL-α-tocopherol (63) and is known to suppress DNA strand scission by hydroxyl radicals produced from UV photolysis of H_2O_2 or its reaction with $Cu(en)_2$ (en, ethylenediamine) (107). It has been reported that oxidative stress may activate transcriptional factors, such NF-kB, through the intracellular signal transduction system (85) and induce expression of proto-oncogenes such as c-fos and c-jun (59). In addition, oxidative stress induces DNA damage, such as modified

base products and strand breaks, that may lead to mutations and chromosomal aberrations. Furthermore, reactive oxygen species can exert suppressive effect on proteinase inhibitors (95), which in turn can induce enhanced proteinase activity and this may facilitate tumor invasion and metastasis. In a recent report (29), elevated levels of matrix metalloproteinase, known to contribute to the invasion and metastasis of various human malignancies, were described in early duct epithelial hyperplasias with increased immunohistochemical stainability from atypical hyperplasias to carcinoma in the pancreas. In fact, an inhibitor of matrix metalloproteinase given in the diet decreased the development of pancreatic carcinoma (29). Thus, oxidative stress could have an important role in pancreatic tumor progression and the antioxidative activity of PCA could therefore be of significance for the influence on invasive pancreatic tumors found in the present study.

In conclusion, dietary administration of PCA after BOP initiation can inhibit progression of pancreatic carcinogenesis in hamsters, although the exact mechanisms remain to be elucidated. The evidence from this study suggests that further research on the potential of PCA for prevention of cancer development is warranted.

2-5 Summary

The chemopreventive effects of protocatechuic acid (PCA) were investigated during the post-initiation stage of the N-nitrosobis(2-oxopropyl)amine (BOP)-initiated hamster pancreatic tumorigenesis model. Female 5-week-old hamsters were divided into 6 groups. Animals in groups 1-3, each consisting of 30 hamsters, were given two s.c. injections of 20 mg/kg body weight of BOP with a one week interval as an initiation treatment. After BOP injection, hamsters in group 1 and 2 were respectively fed diet supplemented with 1000 or 500ppm of PCA for 49 weeks. The animals in group 3 were treated with BOP alone. The animals in group 4-6, each consisting of 10 hamsters, were given 1000 or 500ppm PCA, or basal diet alone without prior BOP injection. At the treatment of experimental week 52, the incidences and multiplicities of neoplastic lesions in the pancreas were comparable among the BOP-treated groups. However, the incidence of pancreatic tumors larger than 3cm was significantly lower in the PCA-treated high dose groups than the control group ($p<0.05$). Moreover the incidence of advanced pancreatic cancers which had directly invaded adjacent tissues such as the diaphragm, spleen and stomach was reduced by the PCA treatments, being significantly ($p<0.01$) lower in group 2 than in group 3. Our results thus indicated that PCA can inhibit the late post-initiation or progression phase of BOP-induced pancreatic carcinogenesis in hamsters.

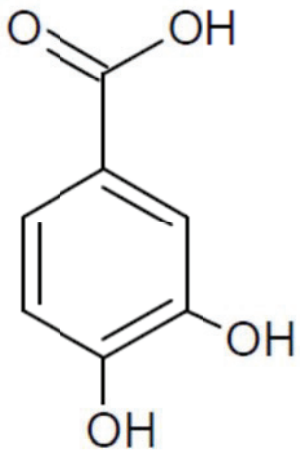

Figure 3. Chemical structure of protocatechuic acid (PCA)

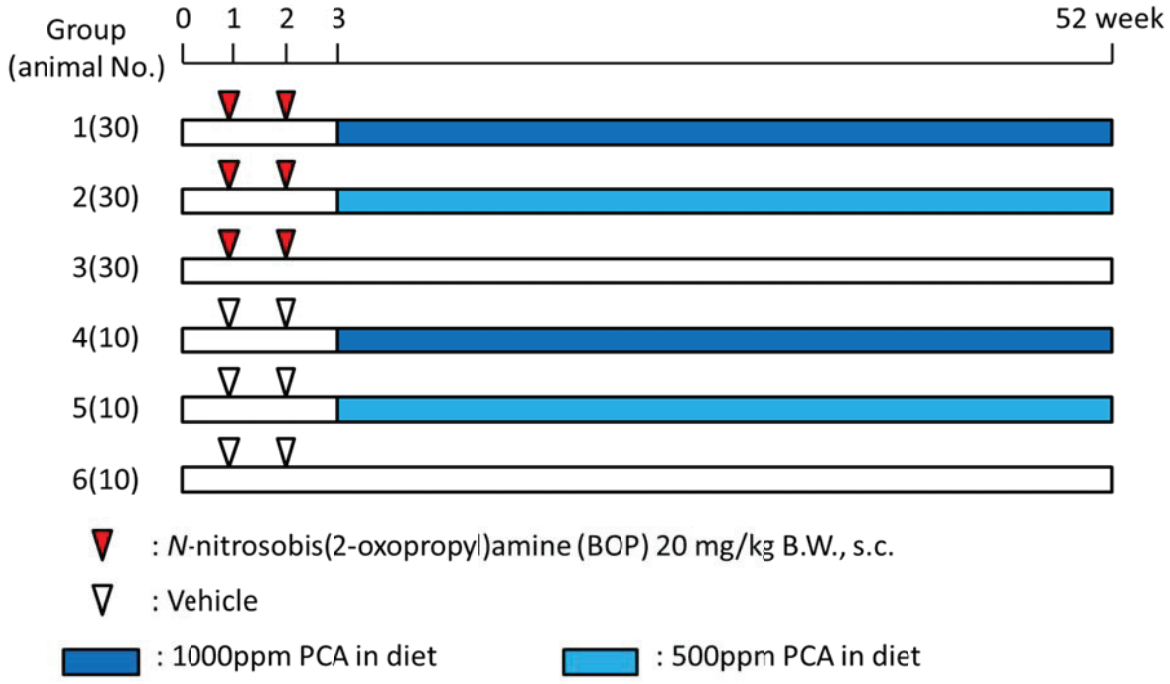

Figure 4. Experimental design

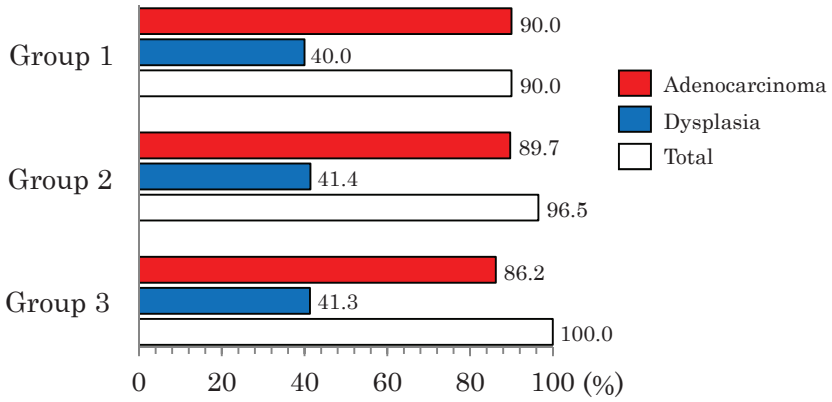

Figure 5. Incidence of pancreatic proliferative lesions

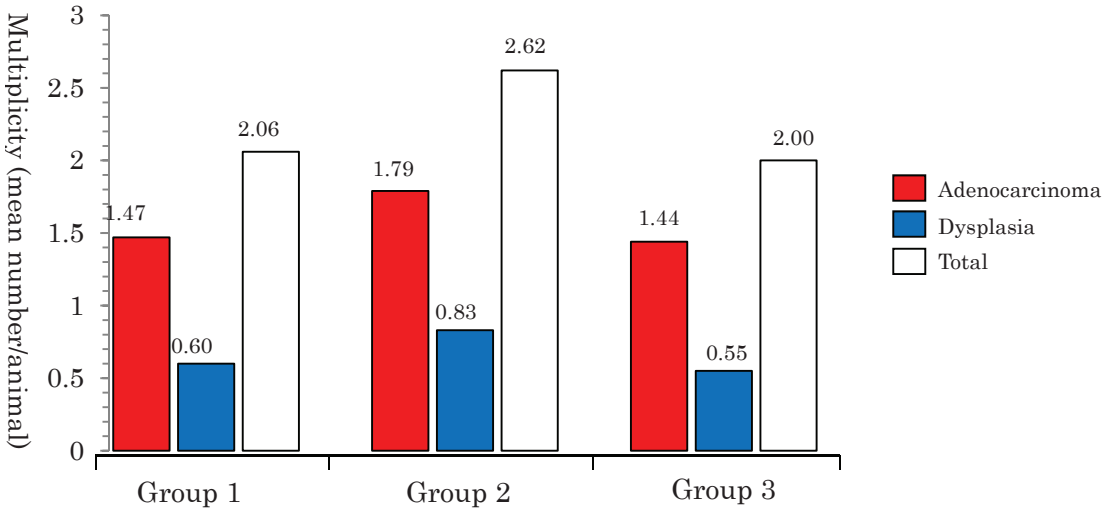

Figure 6. Multiplicity of pancreatic proliferative lesions

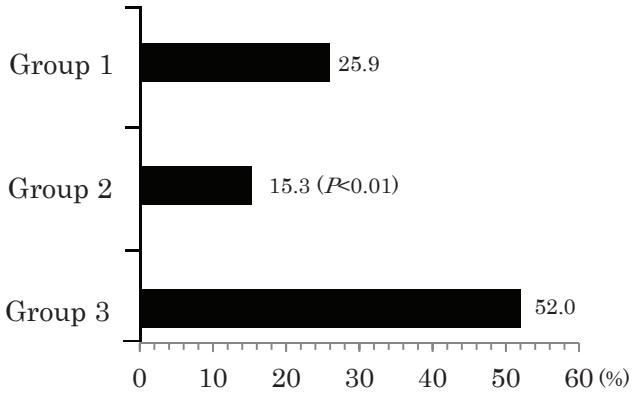

Figure 7. Incidence of invasive pancreatic cancers.

Table 6. Macroscopic findings for BOP-induced pancreatic tumors

	Effective number of hamsters	Number of hamsters with macroscopic pancreatic tumors	Tumor size (diameter)				Total	Number of tumors per hamster
			<1 cm	1-2 cm	2-3 cm	>3 cm		
Group 1	30	22 (73%)	17 (49%)	14 (40%)	4 (11%)	0 (0%)*	35	1.2
Group 2	30	23 (77%)	12 (35%)	6 (18%)	14 (41%)	2 (6%)	34	1.1
Group 3	27	20 (74%)	9 (30%)	9 (30%)	5 (17%)	7 (49%)	30	1.1

* : significantly different from group 3 at $P<0.05$

Table 7. Sites and types of proliferative lesions in other organs.

		Group 1		Group 2		Group 3	
Organ	Lesions	%	No./animal	%	No./animal	%	No./animal
Lung	adenoma	89.3	3.4±2.5 [a)]	86.7	2.1±2.3	83.3	2.9±2.2
	adenocarcinoma	39.3	0.5±0.7	33.3	0.4±0.6	33.3	0.5±0.7
Liver	hepatocellular adenoma	58.6	1.3±1.7	79.3	1.8±1.5	72.4	1.7±1.5
	hepatocellular carcinoma	20.7	0.2±0.4	13.8	0.3±0.6	17.2	0.2±0.4
	cholangiocellular adenoma	34.5	0.6±1.2	27.6	0.6±0.5	31.0	0.5±0.7
	cholangiocellular carcinoma	55.2	0.8±0.8	58.6	0.7±0.7	65.5	0.8±0.7
	hemangioma	6.9	0.1±0.3	0	0	3.4	0.03±0.2
Kidney	adenocarcinoma	3.3	0.03±0.2	6.9	0.1±0.4	6.9	0.1±0.2
Ovary	cystadenoma	3.3	0.03±0.2	3.3	0.03±0.2	3.3	0.03±0.2

[a)] :Mean ± S.D.

Chapter 3

Enhancing Effects of Oltipraz on the Development of Spontaneous Hepatic Lesions in LEC Rats

3-1 Introduction

Oltipraz [4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione](Figure 8), an antischistosomal agent, was first evaluated as a treatment for schistosomiasis in the early 1980s [7]. It acts by reducing glutathione (GSH) stores of parasites, but paradoxically also increases levels of GSH in many tissues of the host [5, 14]. Subsequent studies demonstrated that oltipraz and related 1,2-dithiole-3-thiones are potent inducers of enzymes concerned with maintenance of the reduced GSH pool, as well as enzymes important for electrophile detoxification in tissues of rats and mice [36, 52]. These activities are presumed responsible for its prevention of hepatotoxicity due to CCl4, aflatoxin B1 and acetaminophen in rodents, and its broad range of anti-carcinogenic activity [16, 37, 39, 58]. Oltipraz has recently undergone Phase II trials in the United States to characterize the dose response for biomarker modulation and drug toxicity with chronic administration to humans [30, 38, 111].

LEC rats (Long-Evans rats with a cinnamon-like coat color) were established as a mutant strain displaying hepatic damage with severe jaundice [83], identified as a model of Wilson's disease [47, 48]. Spontaneous hepatic damage appears suddenly in the rats about 3 to 4 months, with a high rate of lethality and hepatoma development, about 1 year after birth. The clinical characteristics resemble those of human fulminant hepatic damage [115], and are caused by Cu accumulation in the liver [47]. Genetic analysis has revealed that a defect in the Cu transporting ATPase gene is responsible for the hepatic damage, similar to Wilson's disease [54, 113].

In the present study, the possible inhibitory effects of Oltipraz on the development of hepatic damage to LEC rats administered diet supplemented with drug at a dose of 400ppm were investigated through evaluation of serum biochemistry and histopathological changes.

3-2 Materials and Methods

Animals

Male LEC rats (5 weeks old) were purchased from Charles River Japan Inc (Hino, Japan) and acclimated for 1 week prior to the initiation of the study. The animals were housed, 5 per polycarbonate cage, in an air-conditioned room at 23±2 C with 60±5% humidity under a daily cycle of alternating 12-hour periods of light and darkness. They had free access to a basal diet Oriental MF (Oriental Yeast Co, Ltd, Tokyo), and tap water throughout the study.

Experimental Design

A total of 35 male 6-week-old LEC rats were divided into 2 groups. One group was fed diet supplemented with 400ppm Oltipraz kindly supplied by Rhone-Poulenc Rorer (France) and another was fed basal diet alone. The dose was selected according to a previous experiment using conventional rats [79], in which the maximum tolerated dose (MTD) was determined to be 500ppm. Therefore, 400ppm (80% MTD) was applied in the present study as in several chemoprevention studies [78,79]. All rats were observed daily for symptoms of toxicity. Body weight and food consumption were measured once every week. Because rats died between the 10th and 11th weeks, at weeks 10, 15, and 25 of the administration, 3 to 5 animals in each group were sacrificed for sampling of blood and tissues. At every sacrifice point, blood samples were taken from the abdominal aorta under ether anesthesia, centrifuged at 3,000 rpm for 10 minutes, and serum parameters including: total protein (T.pro), the albumin/globulin ratio (A/G), albumin, total cholesterol (T.cho), blood urea nitrogen (BUN), creatinine, calcium (Ca), inorganic phosphate (P), iron (Fe), total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ -GTP), cholinesterase (ChE), electrolytes (Na, K, and Cl), copper (Cu), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate

dehydrogenase (LDH) were determined. The animals at each sacrifice point, and those found moribund or dead were also necropsied for pathological examination. At necropsy, the kidney and liver were fixed in acetone, routinely processed for embedding in paraffin, and sections were stained with hematoxylin and eosin.

Assessment of GSH Levels in the Liver and Kidney

Separate male 18-week-old LEC rats were divided into 2 groups. One group was fed basal diet supplemented with 400ppm oltipraz and the other group was fed basal diet alone. After 7 weeks' administration, 3 and 6 rats in the treated and non-treated groups, respectively, were sacrificed for sampling of tissues. Livers and kidneys were analyzed for GSH levels by reaction with o-phthalaldehyde to form a fluorescent product with an emission peak at 420 nm on excitation at 350 nm [27].

Statistical analysis

The data for organ weights, biochemical parameters and GSH levels were examined with the Student's t-test.

3-3 Results

Body weights in the treated group were transiently lower than those in the control group from week 9 to 13 of the oltipraz administration (Figure 9). Body weights at week 15 were significantly ($p < 0.05$) lower in the treatment group than in the control group. In the oltipraz group, 8 animals died or became moribund during treatment weeks 10 and 11. In contrast, only 1 rat in the nontreatment group died after 16 weeks (Figure 9). All dead or moribund animals showed severe or moderate jaundice. Absolute liver and kidney weights were increased or showed a tendency for increase by the oltipraz-treatment at each sacrifice point. In this context, relative liver weights were increased ($p < 0.05$ or 0.01) in the oltipraz-treated group at each sacrifice point, and relative kidney weights were increased ($p < 0.05$) in the treated group at week 25 as compared to the control values (Table 8). Data for serum biochemistry are shown in Table 9.

The parameters related to hepatic toxicity (AST, ALT, γ -GTP, and LDH) were significantly ($p < 0.05$ or 0.01) higher in the oltipraz-treated group than in the control group at week 25. The serum Cu, remarkable in all LEC rats, and ALP were significantly ($p < 0.05$ or 0.01) higher in the oltipraz treated group at each sacrifice point. The serum Fe was significantly ($p < 0.01$) increased in the oltipraz-treated group at weeks 10 and 15, whereas TIBC was significantly ($p < 0.05$) increased at week 25.

Histopathologically, enlarged hepatocytes with large nuclei, pigment granule-laden Kupffer cells, oval cell proliferation, focal necrosis, and subsequent increase of erythroblasts in the liver (Figure 10) as well as hypertrophy of renal tubule cells were observed in both groups as typical lesions of LEC rats [86]. However, severity grades of enlarged hepatocytes with large nuclei, pigment granule-laden Kupffer cells and hypertrophy of renal cells were greater in the treated group after 15 and 25 weeks of administration (Table 8). Likewise, sequential histopathological changes of the liver were well correlated with serum biochemical data. These lesions were also observed in

all moribund or dead rats.

Data for GSH levels are summarized in Figure 11. The levels (nmol/mg protein) in the liver were 74.8 and 45.1 for the oltipraz-treated and non-treatment groups, respectively. Thus, the GSH level in LEC rats given oltipraz was significantly ($p < 0.01$) higher than the non-treatment value. GSH levels in the kidney, however, were not affected at 20.4 and 20.1 for the oltipraz-treated and non-treatment groups, respectively.

3-4 Discussion

The results of the present study clearly indicate that dietary oltipraz administration exacerbates the severity of spontaneous hepatic lesions in LEC rats. Because oral 400ppm administration of oltipraz is 80% MTD to conventional rats [79], the fact that the 400ppm treatment increased the lethality clearly indicates that LEC rats are more susceptible to oltipraz than conventional rats.

It is known that oltipraz has various activities such as: (a) enhancement of GSH synthesis with induction of γ -glutamyl cysteine synthetase, a rate-limiting enzyme of glutathione biosynthesis [15]; (b) increase in levels of mRNAs and activities of Phase II detoxification enzymes (glutathione-S-transferase, NADP(H): quinone oxidoreductase and UDP-glucuronosyl transferase) [4, 15, 38, 39, 41, 54]; and (c) elevated mRNA expression and activities for anti-oxidant enzymes (Mn-superoxide dismutase and catalase) [6]. These activities are recognized as modes of action of various chemopreventive agents. Oltipraz has in fact been well investigated for prevention of hepatotoxicity caused by CCl_4, aflatoxinB1, and acetaminophen, and carcinogenesis due to several carcinogens in rodents [16, 39, 58]. GSH is a very versatile ligand, forming stable complexes with both hard and soft metal ions [46]. This binding could have a protective function against heavy metals. Abnormal copper accumulation in LEC rat livers has been shown to be genetically linked to the development of hepatic damage [93].

From this, we expected that oltipraz might prevent spontaneous hepatic damage of LEC rats by raising GSH synthesis. In the present study, GSH level of the liver was in fact increased in the oltipraz-treated group, but the data for mortality, serum biochemistry, and histopathology rather showed enhancement of the development of hepatic damage in association.

During weeks 10 and 11 of the treatment (16 and 17 weeks of age), moribund or dead animals in the oltipraz-treated group were found with reduced body weight. It is well documented

that Copper rapidly accumulates in the livers of LEC rats during weeks 16 to 28 of age [43], and in this period spontaneous severe hepatic injury appears with a high rate of mortality. Histologic differences between oltipraz-treated and control LEC rats were limited to severity, not character of the lesions, and presumably related to the mechanism of spontaneous hepatic damage in the LEC rats, that is supposed to be primarily due to Cu accumulation.

Copper is known to enhance the generation of reactive oxygen species in various tissues, and one might expect an increased oxidative stress to be associated with hepatic damage in LEC rats. Several studies have suggested that many anti-oxidants such as flavonoids, phenolic compounds, and N-acetyl cysteine can exhibit pro-oxidant behavior in the presence of Cu [49, 62, 82]. It is well known that oltipraz has anti-oxidative activity, modulating free radical-dependent toxicity through both direct and indirect mechanisms [37]. Therefore, oltipraz itself or induced GSH may exert prooxidant potential with accumulation of Cu in LEC rats.

In conclusion, our results indicate that spontaneous hepatic damage in LEC rats is enhanced by oltipraz, by a mechanism that remains to be elucidated.

3-5 Summary

Oltipraz, developed as an antischistosomal agent, protects against the hepatotoxicity of many xenobiotics and is known to be an effective inhibitor of experimental carcinogenesis in rodents. In the present study, we investigated its effects on the development of lesions in LEC rats, established as a mutant strain characterized by a hereditary predisposition for hepatic damage with severe jaundice. A total of 35 male 6-week-old LEC rats were divided into 2 groups, one administered diet supplemented with oltipraz at a dose of 400ppm, and the other fed basal diet alone. Animals in each group were sequentially sacrificed at 10, 15, and 25 weeks after commencement of the oltipraz administration. Eight animals died or became moribund in the oltipraz group during weeks 10 and 11 of the treatment, whereas only one rat in the non-treatment group died after 16 weeks. All dead or moribund animals showed severe or moderate jaundice. The treatment caused a decrease in body weight gain from 9 to 13 weeks, and an increase in relative liver weight at each sacrifice point. Serum biochemical assays performed at week 25 revealed elevated levels of serum AST, ALT, LDH, ALP, γ-GTP, and Cu in the treated-animals. The glutathione level in the livers of oltipraz-treated animals was significantly higher than that in the control rats. Histopathologically, enlarged hepatocytes with large nuclei, focal necrosis, pigment granule-laden Kupffer cells and hypertrophy of renal tubule cells were observed in both groups, but the severity of these changes was greater in the oltipraz group. Our results thus indicate that spontaneous hepatic damage in LEC rats is enhanced by oltipraz, by a mechanism that remains to be elucidated.

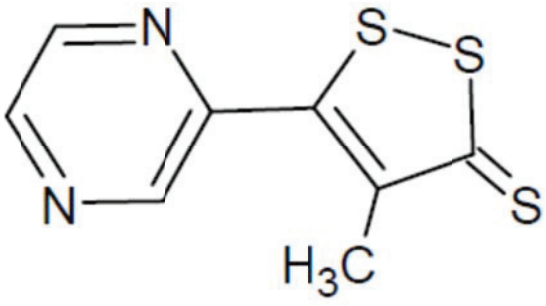

Figure 8. Chemical structure of Ortipraz.

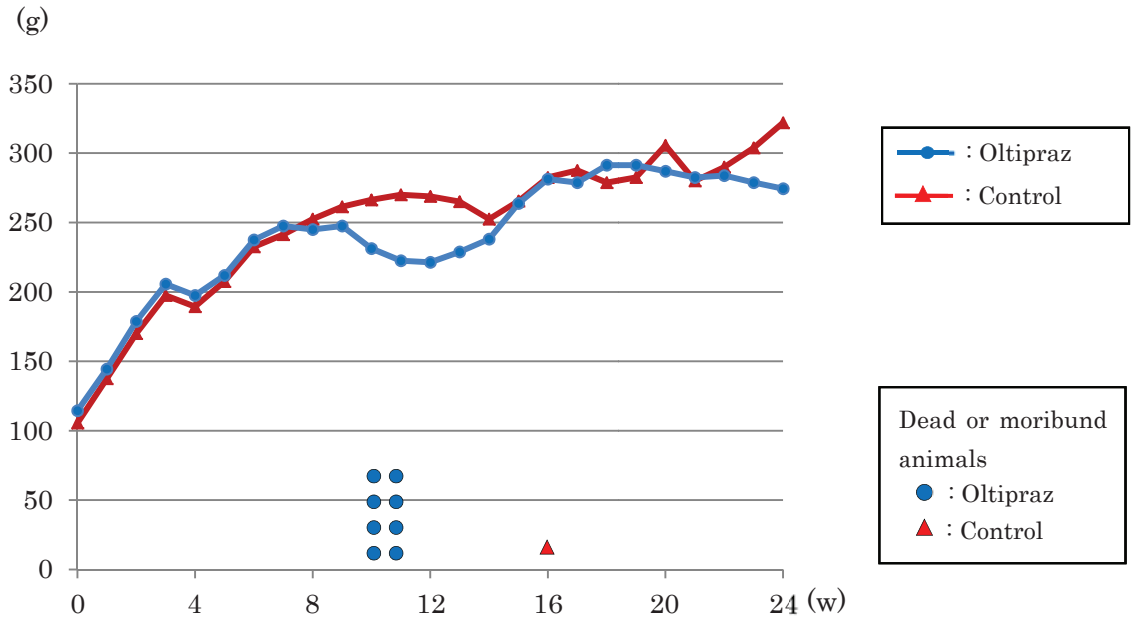

Figure 9. Body weight curves and mortality.

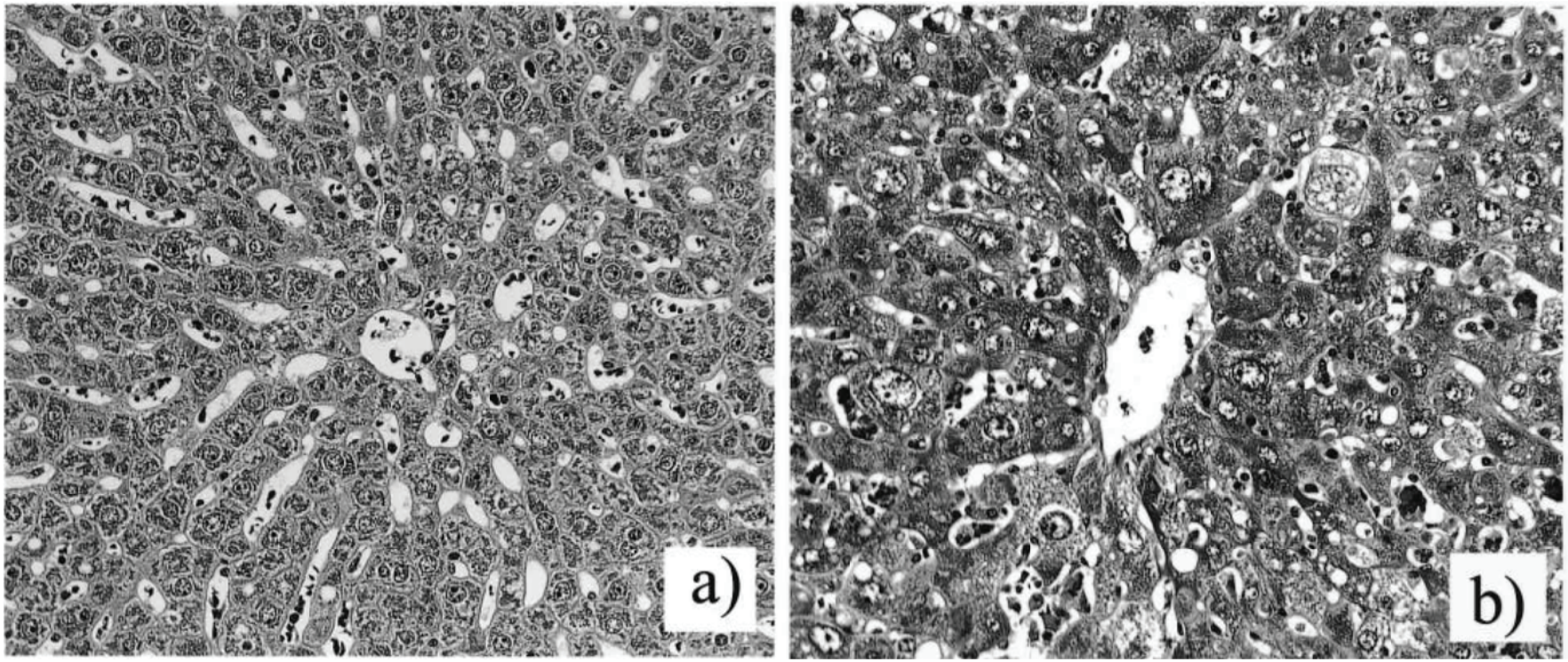

Figure 10. Photomicrographs of the liver in LEC rats in the control group (a), or the oltipraz-treated group (b) at 25 weeks, the latter clearly showing hepatocyte enlargement with large nuclei.

Table 8. Final body weight and relative organ weights

		Number of rats examined	Body weight (BW) (g)	Liver (% BW)	Kidney (%BW)
10w	Oltipraz	5	233±11[a]	3.21±0.34[*]	0.68±0.04
	Control	5	248±13	2.54±0.07	0.67±0.03
15w	Oltipraz	3	220±22[*]	3.09±0.18[*]	0.82±0.28
	Control	5	253±4	2.66±0.13	0.65±0.02
25w	Oltipraz	4	271±22	2.67±0.13[**]	0.67±0.08[*]
	Control	4	295±18	2.09±0.06	0.59±0.02

[a] : Mean±S.D.

[*] : Significantly different from the control group at $P<0.05$.

[**] : Significantly different from the control group at $P<0.01$.

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Table 9. Serum biochemistry

	10 week		15 week		25 week	
	0ppm	400ppm	0ppm	400ppm	0ppm	400ppm
Number of rats examined	5	5	5	2	4	4
T. pro (g/dL)	5.3±0.2[a]	5.2±0.2	5.4±0.1	5.2±0.3	5.1±0.1	5.1±0.2
A/G ratio	5.8±0.5	6.6±1.2	4.5±0.5	4.2±0.3	5.1±0.6	4.8±0.9
albumin (g/dL	4.5±0.2	4.5±0.2	4.4±0.2	4.2±0.3	4.3±0.1	4.2±0.2
T. cho (mh/dL)	57.4±5.6	57.0±5.0	61.8±7.6	49.0±7.1	51.0±1.4	52.3±3.1
BUN (mg/dL)	13.0±1.4	15.5±0.6**	25.0±1.6	21.5±5.2**	19.9±0.5	18.3±0.5**
Creatinine (mg/dL)	0.2±0.0	0.2±0.1	0.3±0.0	0.3±0.0	0.4±0.0	0.3±0.1*
Ca (mg/dL)	9.0±0.2	9.1±0.1	9.3±0.1	9.6±0.3	9.2±0.1	8.8±0.1**
P (mg/dL)	6.0±0.3	6.2±0.7	8.0±0.5	7.5±1.1	9.2±0.1	8.8±0.1
Fe (μg/dL)	115±21	209±36**	172±32	261±44**	154±41	124±69
TIBC (μg/dL)	514±36	531±29	668±37	757±36	567±28	644±43*
UIBC (μg/dL)	400±53	322±55*	496±33	496±8	413±32	469±77
Cu (μg/dL)	10.4±3.8	31.2±14.0*	41.2±19.6	51.0±15.6*	23.0±9.1	67.3±7.6**
AST (IU/L)	134±23	213±94	445±275	252±68	305±150	607±122*
ALT (IU/L)	59±22	385±256*	472±337	305±47*	235±142	59±22
LDH (IU/L)	1676±437	998±303*	1315±780	1374±73	670±315	2919±907**
ALP (IU/L)	317±50	405±45*	423±77	818±310*	267±46	391±53*
γ-GTP (IU/L)	N.D.	6.5±1.3	7.0±4.5	23.0±9.9	6.5±1.7	21.8±1.5**
Che (IU/L)	6.4±1.1	6.6±0.9	8.0±0.8	6.0±1.4	N.D.	7.3±1.3
Na (mEQ/L)	143±1	143±2	138±1	142±3**	145±1	144±1*
Cl (mEQ/L)	110±2	108±2	103±1	106±1	111±1	110±1
K (mEQ/L)	5.0±0.3	4.8±0.2	4.4±0.3	4.5±0.1	4.5±0.2	5.0±0.2*

[a] Mean±SD.

*Significantly different from the control group at $p<0.05$

**Significantly different from the control group at $p<0.05$

N.D.: Not detected.

Table 10. Histopathological findings for LEC rats treated with or without oltipraz.

			Liver													Kideny	
		Number of rats examined	Enlarged hepatocytes with large nuclei [a]				Pigment granules in Kupffer cells [b]		Oval cell proliferation [b]		Focal necrosis [c]			Increase of erythroblast [b]		Hypertrophy of renal cells [b]	
			−	±	+	++	−	+	−	+	−	±	+	−	+	−	+
10w	Oltipraz	5	1	4	0	0	5	0	5	0	2	3	0	5	0	5	0
	Control	5	4	1	0	0	5	0	5	0	4	1	0	5	0	5	0
15w	Oltipraz	3	0	0	2	1	0	3	0	3	0	2	1	2	1	0	3
	Control	5	0	4	1	0	5	0	2	3	0	4	1	5	0	5	0
25w	Oltipraz	4	0	0	0	4	0	4	0	4	0	1	3	2	2	1	3
	Control	4	0	0	4	0	0	4	0	4	0	4	0	3	1	3	1

[a] −, none (or trace); ±, mild; +, moderate; ++, severe.

[b] −, absent; +, prsent.

[a] −, none (or trace); ±, slight; +, moderate.

General discussion

Antioxidant substances are roughly classified into three categories, namely, vitamins, enzymes, and phytochemicals. In this report, three kinds of antioxidants were used in three studies targeting different endpoints.

In the first study, the toxicity of tocotrienols was evaluated by a repeated- dose 90-day oral toxicity study in rats. Tocotrienols are a member of the vitamin E family. Vitamin E is incorporated into the cell membrane where it effectively inhibits peroxidation of lipids. Both tocopherols and tocotrienols can scavenge chain-propagating peroxyl radicals [13, 34]. The antioxidant efficacy of tocotrienols in the cell membrane is higher than that of tocopherols, although their uptake and distribution after oral intake are less than those of alpha-tocopherols [60, 88, 114]. The experiment was conducted using the OECD Test Guideline 408 protocol, "Repeated Dose 90-day Oral Toxicity Study in Rodents" [61], which provides information on the possible health hazards likely to arise from repeated exposure over a prolonged period covering post weaning maturation and growth well into adulthood, as well as information on the major toxicological profiles indicating target organs and the possibility of their accumulation. Therefore, such a repeated-dose study is useful for estimating a no-observed-adverse-effect level (NOAEL), which can be used for selecting doses for chronic or carcinogenicity studies and practically for establishing safety criteria for human exposure. In this study, suppression of body weight increase, decrease in platelet count, slight enlargement of hepatocytes, and reduction of vacuolation in the adrenal cortical region were observed in the high-dosing groups, providing a NOAEL. It was also suggested that these changes may be due to the hypocholestrolemic activity of tocotrienols rather than antioxidative activity [68, 75, 76, 112]. However, in a subsequent combined chronic and carcinogenicity study, nodular hepatocellular hyperplasia with spongiosis or angiectasis was induced even under the NOAEL in a 90-day study [103, 104]. It is likely that a 90-day toxicity study is not sufficient for

predicting chronic toxicity.

In the second study, the modifying effects of protocatechuic acid on proliferative lesions were evaluated using the BOP-initiated hamster pancreatic carcinogenesis model. The modes of antioxidative activity of protocatechuic acid are supposed to be chelating metal transition ions and scavenging free radicals via donating hydrogen atoms or electrons [70]. Thus, protocatechuic acid has been reported to prevent proliferative lesions in a number of cancer-induced models for the colon, liver, urinary bladder, oral cavity, and skin [100]. In this study, dietary protocatechuic acid administration during the post-initiation phase effectively suppressed pancreatic carcinogenesis initiated with BOP as revealed by the reduced size of tumors and decreased incidence of invasive cancers. In chemical carcinogenesis, oxidative stress can not only cause direct damage to DNA, but also modify intercellular communication, protein kinase activity, membrane structure and function, and gene expression resulting in modulation of cell growth. Results of this study suggest that protocatechuic acid inhibits such biological effects caused by oxidative stress in the promoting and progressive phases of carcinogenesis and results in reduced size of tumors and decreased incidence of invasive cancers.

In the third study, the modifying effects of oltipraz on persistent liver injury were evaluated by its administration to LEC rats which accumulate Cu in the liver and develop spontaneous liver lesions. Although oltipraz has been reported to show chemopreventive action in studies under various conditions [28, 16, 40], its administration to LEC rats actually enhanced the development of liver lesions in this study. In terms of oxidative stress in LEC rats, excessive accumulation of Cu in the liver may be unique [47, 48]. In the presence of excessive Cu accumulation, various antioxidants such as flavonoids, phenolic compounds, and N-acetyl cysteine have shown pro-oxidant behavior [49, 65, 82]. It is likely that the liver lesions in LEC rats are not simply controlled by the antioxidant status. Similarly, Kitamura et al. [42] have reported the

modifying effects of several substances on the liver lesions in LEC rats, in which phytic acid and metal chelating agents did not attenuate liver lesions in LEC rats however, N-acetyl cysteine, an enhancer of GSH synthesis, showed an inhibitory action against the development of LEC rat liver lesions. It still remains unclarified when the preventive efficacy of antioxidants is overwhelms the hazardous effects of oxidant stress in LEC rats. Therefore, the duration of experiments may be important for investigating the effects of antioxidants. Further studies are necessary to elucidate the detailed relationship between oxidative-stress-based biological activity in LEC rats and modification by antioxidants.

From the results obtained from these studies, the following are concluded. Adequate study methods are essential for evaluating the potential adverse or beneficial effects of antioxidants. In particular, the results suggest as shown for tocotrienols that conventional 90-day studies may not be sufficient for evaluating the lifetime effects of antioxidants. The BOP-initiated hamster pancreatic carcinogenesis model is a powerful tool for detecting the modifying effects of various chemicals including antioxidants, and histopathological analysis using specific stains for lipid peroxidation may be extremely useful. Finally, as shown in the LEC rat study, the antioxidant oltipraz unexpectedly stimulated the development of spontaneous copper-related liver injury. Again, this indicates that such a study should be based on the mechanism of action.

Acknowledgments

This work was carried out at the Division of Pathology, National Institute of Health Sciences, Tokyo.

This work was not completed without advices and helps of supervisors, and supports of many people.

First of all, I would like to show my grate appreciation to my supervisor, Dr. Akiyoshi Nishikawa (Head of Biological Safety Research Center, National Institute of Health Sciences), for his valuable advices, supports and encouragements throughout these experiments, and in the preparation of this thesis.

I would like to express my deepest gratitude to my main supervisor, Professor Dr. Tokuma Yanai (Laboratory of Veterinary Pathology, Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University), for his valuable advices, supports and encouragement in the preparation of this thesis.

I thank the supervisors, Professor Yoshiyasu Kobayashi (Division of Pathological Science, Department of Basic Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine), Professor Masanobu Goryo (Laboratory of Veterinary Pathology, Veterinary Medicine, Faculty of Agriculture, Iwate University) and Professor Makoto Shibutani (Division of Veterinary Pathology, Department of Veterinary Medicine, Tokyo University of Agriculture and Technology), for their careful reviewing of this manuscript and valuable suggestion.

Finally, I would also like to thank coworkers for my work, Dr. Fumio Furukawa, Dr. Makoto Miyauchi, Dr. Hwa-Young Son, Dr. Takayoshi Imazawa, Dr, Kenichiro Kasahara, and Dr. Kazushi Okazaki, for kind and technical supports.

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