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Suppression of 8-Oxo-2′-deoxyguanosine Formation and Carcinogenesis Induced by *N*-Nitrosobis (2-oxopropyl)amine in Hamsters by Esculetin and Esculin

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Effects of esculetin (6,7-dihydroxycoumarin) and its glycoside, esculin, on 8-oxo-2′-deoxyguanosine (8-oxodG) formation and carcinogenesis induced by a chemical carcinogen, *N*-nitrosobis(2-oxopropyl)amine (BOP), were examined in the pancreas of female Syrian golden hamsters. Animals were administered esculetin by gastric intubation into the stomach 30 min before BOP administration or ingestion of a diet containing esculin for 7 days before BOP administration, and killed 1 or 4 h after BOP treatment, and the contents of thiobarbituric acid-reacting substrates (TBARS) and 8-oxodG in the pancreas were determined. Both compounds suppressed significantly the BOP-induced increases in 8-oxodG and TBARS contents in hamster pancreas. We further investigated the effect of esculin on pancreatic carcinogenesis by the rapid production model induced by augmentation pressure with a choline-deficient diet, ethionine, methionine and BOP. Esculin was given *ad libitum* as a 0.05% aqueous solution in either the initiation or promotion phases. The incidence of invasive tumors in animals given esculin during the initiation phase was significantly smaller than in the control group, while esculin given during the promotion phase showed no apparent effects. These results suggest that the intake of esculin has an inhibitory effect on BOP-induced oxidative DNA damage and carcinogenesis in hamster pancreas.

Keywords: Oxidative DNA damage; Carcinogenesis; Pancreas; 8-Oxo-2′-deoxyguanosine; Esculetin; Esculin

Abbreviations: BOP, *N*-nitrosobis(2-oxopropyl)amine; CD, choline-deficient; 8-oxodG, 8-oxo-2′-deoxyguanosine; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reacting substrates; UV, ultraviolet; HPLC, high-performance liquid chromatography; ECD, electrochemical detector; dG, 2′-deoxyguanosine

INTRODUCTION

N-Nitrosobis(2-oxopropyl)amine (BOP) is known to induce carcinogenesis in hamster pancreas.[1] It is considered particularly advantageous for assessing the modifying effects of chemicals on pancreatic carcinogenicity because the induced carcinomas resemble histologically and biologically those observed in humans.[2] A rapid production model for pancreatic carcinomas has been established by augmentation pressure.[3] The term "augmentation pressure" has been adopted for the promotion procedure that consists of a choline-deficient (CD) diet combined with DL-ethionine and L-methionine and BOP treatment after BOP initiation.[3]

Oxidative damage to DNA is recognized to play a role in the process of carcinogenesis. The formation of 8-oxo-2′-deoxyguanosine (8-oxodG) is thought to reflect oxidative damage to DNA and is the most abundant among more than 20 different species formed by oxidative stress.[4]. The 8-oxodG content is increased by reactive oxygen species (ROS) such as hydroxyl radicals and singlet oxygen. The 8-oxodG content has been found to increase in target organs of animals treated with carcinogens such as nitrosoamines.[5,6]

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It has also been reported that BOP induces the formation of 8-oxodG and thiobarbituric acid reactive substances (TBARS) in hamster pancreas.[7]

Coumarins comprise a group of natural compounds widely distributed in plants,[8] and have recently attracted much attention because of their broad biological activities. Esculetin (6,7-dihydroxycoumarin) is known to be a 5- and 12-lipoxygenase inhibitor and to inhibit the production of leukotrienes and 5-hydroxyeicosatetraenoic acid through the lipoxygenase pathway.[9] Esculetin also shows scavenging activity against ROS, such as superoxide radicals[10] and hydroxyl radicals,[11] and inhibits lipid peroxidation in rat liver.[12] We have recently observed that esculetin and its glycoside, esculin, suppress the formation of 8-oxodG in DNA in cultured human diploid fibroblasts treated with linoleic acid hydroperoxide and ferric ion.[13] The conversion of esculin to esculetin has been observed during the incubation of the cells with esculin.[14] Furthermore, it has been reported that esculetin inhibits the proliferation of mammary tumor cells.[15,16] In this paper, we investigated the inhibitory effects of esculetin and esculin on oxidative damage induced by a single administration of BOP and on pancreatitis formation and carcinogenesis caused by augmentation pressure in the pancreas of Syrian golden hamsters.

MATERIALS AND METHODS

Animals and Chemicals

Female Syrian golden hamsters were obtained at 6 weeks of age from Japan SLC (Shizuoka, Japan), and were fed *ad libitum* a commercial laboratory diet, Oriental MF (Oriental Yeast Inc., Tokyo, Japan), and tap water. The CD diet was obtained from Funabashi Farm Co. (Chiba, Japan). Esculetin and esculin were purchased from Aldrich Chemical Co. (Milwaukee, WI), BOP from Nacalai Tesque Inc. (Kyoto, Japan), and 8-oxodG from Wako Pure Chemicals Industries (Osaka, Japan). The water used for the isolation and hydrolysis of DNA was purified through a Millipore Milli Q system (Millipore Co., Bedford, MA) and then treated with Chelex 100 resin (Bio Rad Laboratory, Hercules, CA) to remove trace amounts of transient metal ions such as iron ion.

Effects of Esculetin and Esculin on Oxidative DNA Damage Caused by a Single Administration of BOP

In experiment 1, animals were divided into five groups consisting of 15 animals each. Animals in groups 3, 4 and 5 received 1% ethanol solution of esculetin at doses of 1.0 (0.18), 2.0 (0.36) and 4.0 (0.71) μmol (mg)/kg body weight to the stomach directly by gastric intubation, animals in groups 1 and 2 received 1% ethanol solution alone. Thirty minutes after BOP administration, animals in groups 2 through 5 were injected subcutaneously with BOP at a dose of 20 mg/kg body weight, animals in group 1 were injected with saline alone. In experiment 2, animals were divided into four groups consisting of 10 animals each. Animals in groups 3 and 4 were fed *ad libitum* a diet containing 0.1 and 1.0% esculin, respectively, for 7 days, while animals in groups 1 and 2 were fed the usual diet. Then, the animals in groups 2 through 4 were injected subcutaneously with 20 mg/kg of BOP, while animals in group 1 were injected with saline. In both experiments, animals were killed by hemorrhage from the abdominal aorta under anesthesia 1 or 4 h after BOP injection for the measurement of TBARS and 8-oxodG, respectively. Pancreases and livers are extirpated and stored at −80°C until analysis.

Effect of Esculin on Pancreatitis Formation and Carcinogenesis Caused by a Choline-deficient Diet, DL-Ethionine, L-Methionine and BOP

The rapid production model for hamster pancreatic carcinomas was performed according to the published protocol.[3] That is, animals were divided into three groups each comprising 20–22 animals. All groups received BOP at a dose of 70 mg/kg body weight as the initiation step, and were then exposed to three cycles of augmentation pressure 11 days after BOP initiation. The augmentation pressure consisted of a choline-deficient diet combined with four daily intraperitoneal injections of 500 mg/kg ethionine and a single intraperitoneal injection of 800 mg/kg methionine followed by a single injection of 20 mg/kg BOP on day 5, after the beginning of the augmentation pressure cycle. Esculin was dissolved in drinking water to prepare a 0.05% (w/v) solution. Animals in group 1 were given the esculin solution during the initiation phase (from 7 days before the initiation dose of BOP to the start of the first augmentation pressure). Animals in group 2 were given the esculin solution during the promotion phase (during the three rounds of augmentation pressure). Animals in group 3 were controls injected subcutaneously with equal volumes of saline. All animals were killed under pentobarbital anesthesia on day 70 after the beginning of the experiment. Body weight was recorded weekly throughout the experimental period.

Determination of Lipid Peroxidation

The level of lipid peroxidation was estimated from the TBARS levels determined by the method of Kosugi *et al.*[17] Protein content was determined by the method of Bradford.[18]

Isolation of Nuclear DNA

Tissues (ca. 150 mg) were homogenized in a glass-Teflon homogenizer in 10 mM Tris–HCl (pH 7.5) containing 0.3 M sucrose, 0.1 mM deferoxamine mesylate, 1% Triton X-100 and 10 mM $MgCl_2$. Homogenates were centrifuged at 10,000*g* for 20 s under an argon atmosphere to remove the cytosolic fraction containing mitochondria. The pellets were washed twice with saline, suspended in a mixture of 1 μl proteinase K solution (1 mg/ml) and 200 μl, 1% SDS/1 mM EDTA (pH 8.0), and incubated at 37°C for 30 min under an argon atmosphere. Then, 300 μl, 7 M NaI and 500 μl isopropyl alcohol were added, and the resulting solution was centrifuged at 14,000*g* for 10 min at 4°C after cooling at −20°C for 10 min under an argon atmosphere. DNA pellets were rinsed with 40% isopropyl alcohol and centrifuged at 14,000*g* for 10 min at 4°C under an argon atmosphere. Then, the DNA pellets were rinsed again with 70% ethanol, centrifuged at 14,000*g* for 10 min at 4°C under an argon atmosphere, and dissolved in 200 μl, 0.01 × SSC/1 mM EDTA buffer (pH 8.0). Ribonucleases T1 (2.5 U) and A (5 μg) were added to the crude DNA solution, and the mixture were incubated at 37°C for 10 min under an argon atmosphere. The samples were then mixed with a mixture (300 μl) of chloroform and isoamyl alcohol (24:1, v/v) under an argon atmosphere, and the mixture was centrifuged at 14,000*g* for 10 min at 4°C. The aqueous phase was transferred to another tube, mixed with 300 μl, 7 M NaI and 500 μl isopropyl alcohol, and the mixture was kept at −20°C for 10 min under an argon atmosphere. The mixture was then centrifuged at 14,000*g* for 10 min at 4°C and the DNA pellet was rinsed with 40% isopropyl alcohol and 70% ethanol as described above and dissolved in 50 μl water treated with Chelex 100 resin. The amount and purity of DNA were determined by UV absorption as described previously.[19]

Determination of Oxidative DNA Damage

DNA (50 μg) was digested with nuclease P1 (2 μg) in 20 mM sodium acetate buffer (pH 4.8) at 37°C for 30 min under an argon atmosphere, and then with alkaline phosphatase (0.65 U) in 100 mM Tris–HCl buffer (pH 7.5) at 37°C for 30 min under an argon atmosphere. The resulting mixture was filtered through an Ultrafree-MC filter (Millipore Co., Bedford, MA), and the filtrate was applied to a high-performance liquid chromatography (HPLC) system with a Symmetry C18 column (4.6 × 100 mm; Waters Co., Milford, MA) and a Coulochem II 5200 electrochemical detector (ESA, Bedford, MA) with a guard cell 5020 and an analytical cell 5011. The mobile phase consisted of citrate buffer (12.5 mM, pH 5.1) and methanol (93:7, v/v) at a flow rate of 0.8 ml/min. 8-oxodG was measured by electrochemical detection (ECD) at an oxidation potential of 350 mV. The 8-oxodG content is expressed as the molar ratio of 8-oxodG to 10^5 2′-deoxyguanosine (dG). The amount of dG was calculated from the absorption at 260 nm in the same measurement.

Histological Examination

Pancreas tissues were fixed in 10% formalin and embedded in paraffin. The samples were sliced at a thickness of 5 μm and stained with hematoxylin and eosin for histopathological examination. Pancreatitis[20] and pancreatic carcinomas[21] were morphologically diagnosed as described previously. All samples were blinded and scored by one person.

Statistic Analysis

Body weight and oxidative damage data are expressed as mean ± SD. Statistical comparisons among groups were carried out by ANOVA followed by Scheffe's *F*-test. Incidence and multiplicity data were analyzed by the χ^2 test and Student's *t*-test, respectively. Differences between groups were considered significant when the probability (*p*) values were less than 0.05.

RESULTS

Effect of Esculetin and Esculin on Oxidative Damage Caused by a Single Administration of BOP

Esculetin and esculin were supplied by gastric intubation as a 1% ethanol solution (experiment 1)

TABLE I Intake of esculetin and esculin by gastric intubation or taking diet containing esculin

Experiment	Dose of coumarin	Period of dose	Intake of coumarin (mg/kg BW/day)
Esculetin (FW, 178.15)	0	30 min	0
	1 μmol/kg	30 min	0.18
	2	30 min	0.36
	4	30 min	0.71
Esculin (FW, 358.25)	0	7 days	0
	0.1%	7 days	6.27*
	1.0	7 days	58.9*

*Intake of esculin per day was calculated from the amount of diet consumed in 24 h and the body weights of the animals.

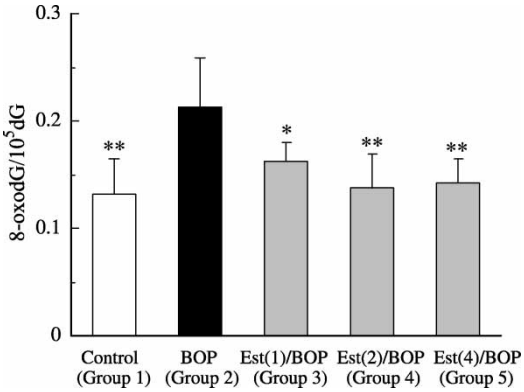

FIGURE 1 Suppression of BOP-induced 8-oxodG formation in the nuclear DNA of hamster pancreas by gastric intubation of esculetin. Animals were supplied with 1.0–4.0 μmol/kg esculetin by gastric intubation 30 min before the subcutaneous administration of BOP (20 mg/kg). Animals were killed 4 h after BOP administration, and the pancreases were removed. Nuclear DNA was isolated and 8-oxodG contents in nuclear DNA were measured by HPLC-ECD as described in "Materials and Methods" section. The data in each column are presented as the mean ± SD of 15 animals. Est(1): 1 μmol/kg esculetin, Est(2): 2 μmol/kg esculetin, Est(4): 4 μmol/kg esculetin, $*p < 0.05$ vs. BOP, $**p < 0.01$ vs. BOP.

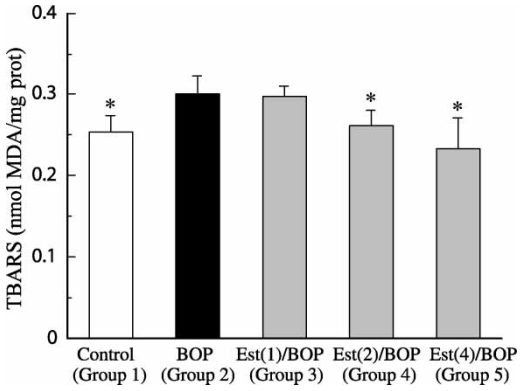

FIGURE 2 Suppression of BOP-induced TBARS formation in hamster pancreas by gastric intubation of esculetin. Animals were treated as described in the legend to Fig. 1 and killed 1 h after BOP treatment. The pancreases were homogenized and TBARS levels and protein contents were measured as described in "Materials and Methods" section. The data in each column are presented as the mean ± SD of 15 animals. Est(1): 1 μmol/kg esculetin, Est(2): 2 μmol/kg esculetin, Est(4): 4 μmol/kg esculetin, $*p < 0.05$ vs. BOP.

or by dietary intake (experiment 2). The dose of esculin per day in experiment 2 was calculated from the amount of diet consumed in 24 h and the body weights of the animals (Table I). We have previously found that the 8-oxodG contents in the nuclear DNA of hamster pancreas reach a maximal value between 2 and 6 h after BOP administration;[7] thus the 8-oxodG contents were measured 4 h after BOP injection in this study. On the other hand, the TBARS contents were assayed 1 h after BOP administration, since high TBARS contents have been observed between 1 and 2 h after BOP administration.[7]

In experiment 1, a single administration of BOP (group 2) to female Syrian golden hamsters significantly increased the 8-oxodG contents in pancreas compared to animals administered saline (group 1) as shown in Fig. 1. Gastric intubation of a 1% ethanol solution alone or 4.0 μmol/kg of esculetin did not increase the contents of 8-oxodG and TBARS (data not shown). Administration of esculetin at doses of 2.0 and 4.0 μmol/kg (groups 4 and 5, respectively) significantly inhibited the increase in 8-oxodG content induced by BOP ($p < 0.01$). Treatment with esculetin at a dose as low as 1.0 μmol/kg esculetin (group 3) induced a significant decrease in 8-oxodG content ($p < 0.05$). Treatment with esculetin at doses of 2.0 and 4.0 μmol/kg similarly suppressed the increase in TBARS content in the pancreas induced by BOP treatment (Fig. 2). On the other hand, the levels of 8-oxodG and TBARS in the liver were not influenced by a single administration of BOP (data not shown).

Esculin was supplied *ad libitum* by mixing in the diet for 7 days in experiment 2. Intakes of esculin from diets containing 0.1 and 1.0% esculin were estimated to be 6.27 and 58.0 mg/kg body weight/day bases on the amount of diet consumed in 24 h. Treatment with a diet containing more than 0.1% esculin for 7 days before BOP administration inhibited significantly the increases in 8-oxodG and TBARS contents induced by BOP (Figs. 3 and 4).

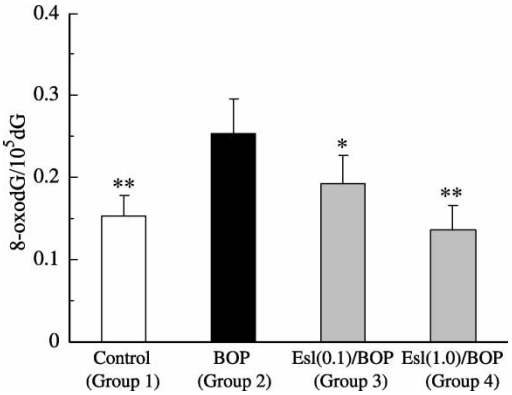

FIGURE 3 Suppression of BOP-induced 8-oxodG formation in the nuclear DNA of the pancreases of hamsters fed a diet containing esculin. Animals were supplied with a diet containing 0.1 or 1.0% esculin or a usual diet for 7 days before BOP administration (20 mg/kg). Animals were killed 4 h after BOP administration, and treated as described in the legend to Fig. 1. The data in each column are presented as the mean ± SD of 10 animals. Esl(0.1): 0.1% esculin, Esl(1.0): 1.0% esculin, $*p < 0.05$ vs. BOP, $**p < 0.01$ vs. BOP.

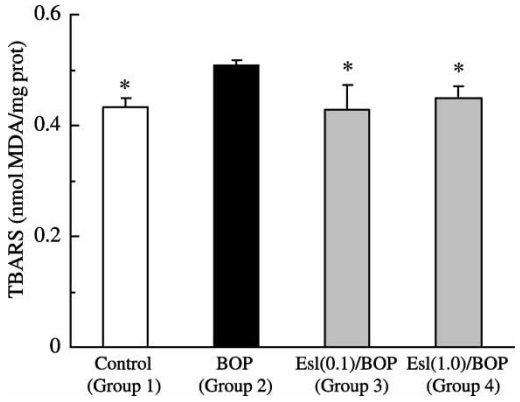

FIGURE 4 Suppression of BOP-induced TBARS formation in the pancreases of hamster fed a diet containing esculin. Animals were treated as described in the legend to Fig. 3. The data in each column are presented as the mean ± SD of 10 animals. Esl(0.1): 0.1% esculin, Esl(1.0): 1.0% esculin, *$p < 0.05$ vs. BOP.

Effect of Esculin on Pancreatitis Formation and Carcinogenesis Caused by a Choline-deficient Diet, DL-Ethionine, L-Methionine and BOP

Body weights and pancreas wet weights did not differ significantly among each group throughout the experimental period (Table II). The incidence of grossly visible tumors was decreased by the ingestion of esculin during the initiation phase as shown in Table III. The incidence of total invasive carcinomas (gross tumors and invasive duct adenocarcinomas) also decreased, while the incidence of invasive duct adenocarcinomas was suppressed, but not significantly. Ingestion of esculin during the promotion phase decreased the incidence of both gross tumors and invasive duct adenocarcinomas, but the changes were not significant (Table III). Incidences of non-invasive carcinomas, such as microcarcinomas and carcinomas *in situ*, were not suppressed by the ingestion of esculin in either the initiation or promotion phases. Multiplicity, mean numbers of carcinomas per hamster, showed a tendency to decrease by the ingestion of esculin in either the initiation or promotion phase. Lesions, such as inter- and intralobule edema, vacuolation, and so on, were observed as symptoms of pancreatitis. Esculin intake during the initiation phase tended to suppress the progression of pancreatitis (Table IV) when the progressive degree of pancreatitis was estimated from the devastation to acinar cells.

DISCUSSION

The incidence of pancreatic cancer is increasing in the world, making it important to investigate the chemoprevention of pancreatic cancer. BOP is a nitrosoamine compound known to be a chemical carcinogen in the pancreas of Syrian golden hamsters. BOP has been reported to undergo reduction to form *N*-nitrosobis(2-hydroxypropyl)(2-oxopropyl)amine, *N*-nitroso(2-hydroxypropyl)amine, their glucuronide- and sulfate-conjugates, and so on;[22] however, metabolites that generate ROS have not been identified at this point. BOP appears to induce oxidative damage to the pancreas through the formation of reactive species during its metabolism. We have reported that BOP increases the 8-oxodG content in the nuclear DNA of hamster pancreas.[7] The 8-oxodG residues in DNA lead to misreading during DNA replication,[23,24] so that the presence of 8-oxodG induces mutagenesis.

Natural phenolic compounds have recently been noted to protect against a wide variety of diseases and the aging process. Esculetin, a natural polyphenolic coumarin, has long been known to be an inhibitor of lipoxygenase,[9] and, further, to show scavenging effects on ROS. Recently, we found that esculetin suppresses the increase in 8-oxodG content in the DNA of TIG-7 cells treated with linoleic acid hydroperoxide and ferric ion.[13] The metabolism of coumarins such as esculetin and esculin has scarcely been elucidated, although catechol derivatives such as flavonoids and catechins have been reported to undergo conjugation, *O*-methylation, and so on.[25,26]

Guanine moieties in DNA are often oxidized during isolation from tissues, and during the hydrolysis of DNA to produce nucleosides for HPLC analysis.[27–29] We employed a method that uses NaI and isopropyl alcohol instead of phenol, sodium chloride and ethanol for the precipitation of DNA, an argon atmosphere during incubation with enzymes, water treated with Chelex 100 resin

TABLE II Body and pancreas weights of hamsters treated according to the rapid production model for pancreatic carcinogenesis

		Body weight (g)					
			Augmentation stress				
Group	No. of animals	Initiation	Start	End	Final weight	Final weight of Pancreas (g)	Intake of esculin (mg/kg BW/day)
Group 1 (Initiation)	22	94.9 ± 13.5	90.8 ± 10.1	93.2 ± 9.6	114.2 ± 15.2	0.44 ± 0.08	52.2
Group 2 (Promotion)	20	98.7 ± 7.4	92.9 ± 9.2	89.9 ± 7.6	108.4 ± 13.3	0.41 ± 0.05	72.1
Group 3 (Control)	20	99.4 ± 8.5	92.0 ± 14.2	95.4 ± 8.3	105.7 ± 9.4	0.41 ± 0.05	0.0

TABLE III Effect of esculin treatment during the initiation and promotion phases on pancreatic carcinogenesis

Group	No. of animals	No. (%) of hamsters developing pancreatic carcinomas					Multiplicity
		Invasive		Non-invasive		Total carcinomas	
		Gross tumors	Invasive duct adenocarcinomas	Micro-carcinomas	Carcinomas *in situ*		
Group 1 (Initiation)	22	1 (4.5)*	2 (9.1)	3 (13.6)	5 (22.7)	11 (50.0)	0.95 ± 0.97
Group 2 (Promotion)	20	2 (10.0)	3 (13.6)**	3 (15.0)	4 (20.0)	12 (60.0)	1.05 ± 1.05
Group 3 (Control)	20	6 (30.0)	3 (15.0) 5 (25.0) 5 (25.0) 11 (55.0)	2 (10.0)	4 (20.0)	17 (85.0)	1.70 ± 1.42

The incidence and multiplicity of carcinomas were determined in the pancreases of hamsters maintained for 24 days after the 3rd cycle of augmentation pressure. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Group 3 (Control).

to remove trace amounts of iron ions, and brown-colored tubes for light shielding whenever possible.[13,30] In this study, the isolation protocol was slightly changed by using deferoxamine, decreasing the amounts of protenase and ribonucleases, and so on.

A single administration of BOP increased the levels of oxidative damage of nuclear DNA and lipids in hamster pancreas identical to the previous observations.[7,13] The increase in 8-oxodG content in nuclear DNA of pancreas following BOP administration was inhibited by gastric intubation of 1 μmol/kg of esculetin 30 min before BOP administration or by the intake of a 0.1% esculin-containing diet for 7 days before BOP administration (Figs. 1 and 3). Similarly, the intake of esculetin or esculin suppressed the TBARS level as shown in Figs. 2 and 4. Since esculin is hydrolyzed to esculetin during incubation at 37°C in experiments using cultured cells,[14] these results indicate that esculetin derived from the hydrolysis of esculin may inhibit oxidative damage to DNA and lipids by scavenging free radicals as shown previously.[14] However, other mechanisms, such as the induction of repair enzyme activities and the chelation of transient metal ions, can not be eliminated.

In this study, the incidence of invasive tumors, such as gross tumors and invasive duct adenocarcinomas, in hamsters fed a diet containing esculin during the initiation phase was reduced. The incidence of invasive tumors also decreased in hamsters given a diet containing esculin during the promotion phase, however, the decrease was not significant. It has been reported that esculetin inhibits cell proliferation of the TMT-081 rat mammary tumor cell line in a concentration-dependent manner,[15] and that esculetin inhibits tumor proliferation in 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis in female rats.[16] There have been no reports so far that esculin inhibits pancreatic carcinogenesis. Treatment with esculin during either the initiation or promotion phase showed a tendency to decrease the total incidence and multiplicity of pancreatic carcinomas, as well as the progression of pancreatitis. Esculetin is known to scavenge against ROS and to be an inhibitor of lipoxygenase enzymes. Lipoxygenase inhibitors, such as baicalein and nordihydroguaiaretic acid (NDGA), have been reported to inhibit proliferation in human breast cancer cells[31] and gastric cancer cells.[32] Esculetin and esculin may suppress cell proliferation in BOP-induced pancreatic carcinogenesis. Alternatively, an inhibition of inflammation may be related to the anticarcinogenic activity of esculin, since inflammation caused by metabolites of arachidonic acid has been reported to be related to cell proliferation and carcinogenesis.[33] Furthermore, it is not excluded the possibility that

TABLE IV Effect of esculin treatment during the initiation and promotion phases on pancreatitis

Group	No. of animal	No. (%) of hamsters developing chronic pancreatitis			
		0%	~10%	~60%	60% ~
Group 1 (Initiation)	22	5 (22.7)	7 (31.8)	10 (45.5)	0 (0.0)
Group 2 (Promotion)	20	3 (15.0)	10 (50.0)	3 (15.0)	4 (20.0)
Group 3 (Control)	20	6 (30.0)	4 (20.0)	5 (25.0)	5 (25.0)

The incidence of pancreatitis was determined in hamsters maintained for 24 days after the 3rd cycle of augmentation pressure. The progressive degree of pancreatitis was estimated from the devastation to acinar cells.

esculetin and esculin reduces the BOP-induced toxicity through induction of metabolizing enzymes such as UDP-glucuronosyltransferases and sulfotransferases.[34]

In conclusion, esculin as well as esculetin inhibit oxidative damage to biological molecules and esculin and, probably, esculetin suppress the initiation phase of pancreatic carcinogenesis. Thus, it is suggested that esculin and esculetin can inhibit pancreatic carcinogenesis. However, further studies are required to elucidate the mechanism of action of the effect of esculetin and esculin on the increase in oxidative damage and pancreatic carcinogenesis induced by BOP administration.

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References

[1] Nishikawa, A., Furukawa, F., Kasahara, D., Tanakamaru, Z., Miyauchi, M., Nakamura, H., Ikeda, T., Imazawa, T. and Hirose, M. (1999) "Failure of phenethyl isothiocyanate to inhibit hamster tumorigenesis induced by *N*-nitrosobis (2-oxopropyl)amine when given during the post-initiation phase", *Cancer Lett.* **141**, 109–115.

[2] Mizumoto, K., Inagaki, T., Koizumi, M., Uemura, M., Ogawa, M., Kitagawa, S., Tsutsumi, M., Toyokawa, M. and Konishi, Y. (1988) "Early pancreatic duct adenocarcinoma", *Hum. Pathol.* **19**, 242–244.

[3] Mizumoto, K., Tsutsumi, M., Denda, A. and Konishi, Y. (1988) "Rapid production of pancreatic carcinoma by initiation with *N*-nitroso-bis(2-oxopropyl)amine and repeated augmentation pressure in hamsters", *J. Natl Cancer Inst.* **80**, 1564–1567.

[4] Hallewell, B. and Arumora, O.L. (1991) "DNA damage by oxygen-derived species: its mechanism and measurement in mammalian systems", *FEBS Lett.* **281**, 9–19.

[5] Chung, F.L. and Xu, Y. (1992) "Increased 8-oxodeoxyguanosine contents in lung DNA of A/J mice and F344 rats treated with the tobacco-specific nitrosamine 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone", *Carcinogenesis* **13**, 1269–1272.

[6] Nakae, D., Kobayashi, Y., Akai, H., Andoh, N., Satoh, H., Ohashi, K., Tsutsumi, M. and Konishi, Y. (1997) "Involvement of 8-hydrocxyguanine formation in the initiation of rat liver carcinogenesis by low dose levels of *N*-nitrosodiethylamine", *Cancer Res.* **57**, 1281–1287.

[7] Takabayashi, F., Harada, N., Tahara, S., Kaneko, T. and Hara, Y. (1997) "Effect of green tea catechins on the amount of 8-hydroxydeoxyguanosine (8-OHdG) in pancreatic and hepatic DNA after a single administration of *N*-nitrosobis (2-oxopropyl)amine (BOP)", *Pancreas* **15**, 109–112.

[8] Egan, D., O'Kennedy, R., Moran, E., Cox, D., Prosser, E. and Thornes, R.D. (1990) "The pharmacology, metabolism, analysis, and applications of coumarin and coumarin-related compounds", *Drug Metab. Rev.* **22**, 503–529.

[9] Neichi, T., Koshihara, Y. and Murota, S. (1983) "Inhibitory effect of esculetin on 5-lipoxygenase and leukotriene biosynthesis", *Biochim. Biophys. Acta* **753**, 130–132.

[10] Chang, W.-S., Lin, C.-C., Chuang, S.-C. and Chiang, H.-C. (1996) "Superoxide anion scavenging effect of coumarins", *Am. J. Chin. Med.* **24**, 11–17.

[11] Hiramoto, K., Ojima, N., Sako, K. and Kikugawa, K. (1996) "Effect of plant phenolics on the formation of the spin-adduct of hydroxyl radical and the DNA strand breaking by hydroxyl radical", *Biol. Pharm. Bull.* **19**, 558–563.

[12] Martín-Aragón, S., Benedí, J.M. and Villar, A.M. (1998) "Effects of the antioxidant (6,7-dihydroxycoumarin) esculetin on the glutathione system and lipid peroxidation in mice", *Gerontology* **44**, 21–25.

[13] Kaneko, T., Tahara, S. and Takabayashi, F. (2003) "Suppression of lipid hydroperoxide-induced oxidative damage to cellular DNA by esculetin", *Biol. Pharm. Bull.* **26**, 840–844.

[14] Kaneko, T., Baba, N. and Matsuo, M. (2003) "Protection of coumarins against linoleic acid hydroperoxide-induced cytotoxicity", *Chem. Biol. Interact.* **142**, 239–254.

[15] Lee, P.P. and Ip, M.M. (1992) "Regulation of proliferation of rat mammary tumor cells by inhibitors of cyclooxygenase and lipoxygenase", *Prostaglandins Leukot. Essent. Fatty Acids* **45**, 21–31.

[16] Kitagawa, H. and Noguchi, M. (1994) "Comparative effects of piroxicam and esculetin on incidence, proliferation, and cell kinetics of mammary carcinomas induced by 7,12-dikethylbenz[*a*]anthracene in rats on high- and low-diets", *Oncology* **51**, 401–410.

[17] Kosugi, H., Kojima, T. and Kikugawa, K. (1991) "Characteristics of the thiobarbituric acid reactivity of oxidized fats and oils", *J. Am. Oil Chem. Soc.* **68**, 51–55.

[18] Bradford, M.M. (1976) "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", *Anal. Biochem.* **72**, 248–254.

[19] Kaneko, T., Tahara, S. and Matsuo, M. (1996) "Non-linear accumulation of 8-hydroxy-2′-deoxyguanosine, a marker of oxidized DNA damage, during aging", *Mutat. Res.* **316**, 277–285.

[20] Takabayashi, F. and Harada, N. (1995) "Effects of green tea catechins (Polyphenon 100) on DL-ethionine-induced acute pancreatitis", *Pancreas* **11**, 127–131.

[21] Pour, P.M. and Wilson, R.B. (1980) "Experimental tumors of the pancreas", In: Moosa, A.R., ed, *Tumors of the Pancreas* (Williams and Wilkins, Baltimore), pp 37–58.

[22] Kokkinakis, D.M., Scarpelli, D.G., Subbarao, V. and Hollenberg, P.F. (1987) "Species differences in the metabolism of *N*-nitroso(2-hydroxypropyl)(2-oxopropyl)amine", *Carcinogenesis* **8**, 295–303.

[23] Shibutani, S., Takeshita, M. and Grollman, A.P. (1991) "Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG", *Nature* **349**, 431–433.

[24] Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S. and Loeb, L.A. (1992) "8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G–T and A–C substitutions", *J. Biol. Chem.* **267**, 166–172.

[25] Terao, J. (1999) "Dietary flavonoids as antioxidants *in vivo*: conjugated metabolites of (−)-epicatechin and quercetin

participate in antioxidative defense in blood plasma", *J. Med. Investig.* **46**, 159–168.
[26] Shimoi, K., Okada, H., Furugori, M., Goda, T., Takase, S., Suzuki, M., Hara, Y., Yamamoto, H. and Kinae, N. (1998) "Intestinal absorption of luteolin and luteolin 7-*O*-β-glucoside in rats and humans", *FEBS Lett.* **438**, 220–224.
[27] Claycamp, H.G. (1992) "Phenol sensitization of DNA to subsequent oxidative damage in 8-hydroxyguanine assays", *Carcinogenesis* **13**, 1289–1292.
[28] Helbock, H.J., Beckman, K.B., Shigenaga, M.K., Walter, P.B., Woodall, A.A., Yeo, H.C. and Ames, B.N. (1998) "DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine", *Proc. Natl Acad. Sci. USA* **95**, 288–293.
[29] Hamilton, M.L., Guo, Z.M., Fuller, C.D., Van Remmen, H., Ward, W.F., Austad, S.N., Troyer, D.A., Thompson, I. and Richardson, A. (2001) "A reliable assessment of 8-oxo-2-deoxyguanosine levels in nuclear and mitochondrial DNA using the sodium iodide method to isolate DNA", *Nuclear Acids Res.* **29**, 2117–2126.
[30] Kaneko, T. and Tahara, S. (2000) "Formation of 8-oxo-2′- deoxyguanosine in the DNA of human diploid fibroblasts by treatment with linoleic acid hydroperoxide and ferric ion", *Lipids* **35**, 961–965.
[31] Tong, W.G., Ding, X.Z. and Adrian, T.E. (2002) "The mechanisms of lipoxygenase inhibitor-induced apoptosis in human breast cancer cells", *Biochem. Biophys. Res. Commun.* **296**, 942–948.
[32] Wong, B.C.Y., Wang, W.P., Cho, C.H., Fan, X.M., Lin, M.C.M., Kung, H.F. and Lam, S.K. (2001) "12-Lipoxygenase inhibition induced apoptosis in human gastric cancer cells", *Carcinogenesis* **22**, 1349–1354.
[33] Lewis, J.G. and Adams, D.O. (1987) "Inflammation, oxidative DNA damage, and carcinogenesis", *Environ. Health Perspect.* **76**, 19–27.
[34] Furukawa, F., Nishikawa, A., Chihara, T., Shimpo, K., Beppu, H., Kuzuya, H., Lee, I.S., Hirose, M., "Chemopreventive effects of *Aloe arborescens* on *N*-nitrosobis(2-oxopropyl)amine-induced pancreatic carcinogenesis in hamsters", *Cancer Lett.* **178**, 117–122.