The Effect of the Wine on Mutagenicities of Some Mutagenic Substances in the *Salmonella*/Microsome

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Summary

The white wine and the red wine condensates were examined for antimutagenic activity with *Salmonella typhimurium* test strain, TA98. The red wine significantly decreased the reverse mutation induced by crude dimethyl sulfoxide extracts of grilled beef, and by cigarette smoke condensate and activated Trp-P-1 in the presence of a rat liver microsomal activation system. Furthermore, the red wine condensate significantly decreased the mutation induced by dimethyl sulfoxide extract of gasoline engine exhaust. The white wine condensate had no significant antimutagenic activity on all mutagenic substances except for activated Trp-P-1.

Introduction

It has been epidemiologically suggested that there are many environmental factors related to human carcinogenesis. The development of screening methods for environmental carcinogens by using their mutagenicity[1] has enabled various types of mutagens/carcinogens to be detected and identified in daily foods; nitrosoamines,[2,3]

Abbreviations: Trp-P-1, 3-amino-1,4-dimetyl-5H-pyrido-(4,3-b)-indol; N-OH- Trp-P-1, 3-hydroxyamino-1,4-dimetyl-5H-pyrido-(4,3-b)indol; Trp-P-2, 3-amino-1-metyl-5H-pyrido-(4,3-b)indol;AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; EGB, extract of grilled beef; CSC, cigarette smoke condensate; EDE, extract of diesel engine exhaust; EGE, extract of gasoline engine exhaust.

mycotoxins,[4] flavonoid compounds,[5,6] pesticides,[7] artificial food additives,[7,8] and many heterocyclic amines.[9-15] Some of these substances have been found to be generated during the storage, cooking, and digestion of foods. Trp-P-1 and Trp-P-2 are mutagenic pyrolysates of tryptophan origin, which were first isolated from broiled beef and demonstrated to be carcinogenic in rats.[11,12] However, little is known about the combined effects of these mutagens/carcinogens and various food components when taken together. We take in many kinds of compounds together in our diet every day, and biochemical reactions within the body usually with numerous coexisting compounds. In previous papers,[16,17] the present author reported the synergism of indigocarmine, an artificial food dye, with Trp-P-1 and Trp-P-2. Further, we investigated the effects of many kinds of foodstuffs on the mutagenicity of dimethyl sulfoxide extracts of grilled beef, and found that some of them significantly decreased the reverse mutations induced by the extracts in the presence of a microsomal activation system.

Since ancient times, the wine has been most drunk in the alcoholic beverage in all over the world. Many workers[18-31] have investigated the beneficial pharmacological and physiological effects of the wine, and it has been suggested that the wine may prevent human heart disease, through epidemiological investigations of diets. Conversely, little is known about antimutagenic and anticarcinogenic activities of the wine. In this paper, the effect of the wine on the mutagenicities of dimethyl sulfoxide extracts of grilled beef and the automobile exhaust, cigarette smoke condensate , activated Trp-P-1(N-OH-Trp-P-1), and AF-2, a nitrofuran compound used as a food additive from 1966 to 1975 only in Japan, were investigated with or without microsomal activation system. In the course of the study, the present author found that only the red wine significantly inhibited the reverse mutations induced by all these mutagenic substances except for the extract of diesel engine exhaust and AF-2.

Materials and Methods

Chemicals. S-9 fraction prepared from rat liver pretreated with phenobarbital and 5, 6-benzoflavone was obtained from Oriental Yeast Co., Ltd. Trp-P-1 and other chemicals used were obtained from Wako Pure Chemical Industries Co. Ltd., the chemicals used being of special grade.

Bacterial strain. Strain TA 98 of *Salmonella typhimurium* were kindly supplied by Dr.B.N.Ames of the University of California. The bacteria were cultured in nutrient

broth for 14h at 37℃ just before the mutation assay.

Mutation assay and inhibition test. The mutation assay was done by a modification of the method of Yahagi *et al.*[32] S-9 mix contained 50 μmol of sodium phosphate buffer (pH 7.4), 4 μmol of magnesium dichloride, 16.5 μmol of potassium chloride, 2.5 μmol of glucose-6-phosphate, 2 μmol of NADH, 2 μmol of NADPH, and 50 μl of S-9 mix fraction in a total volume of 0.5 ml. Mutagenicity was tested on TA98 with or without S-9 mix according to the type of each mutagen. For the inhibition test, 0.1 ml of each mutagen, 0.1 ml of the wine condensate and 0.5 ml S-9 mix or phosphate buffer were simultaneously incubated with 0.1 ml of bacterial suspension at 37℃ for 20 min and poured on minimal-glucose agar plates with 2 ml of soft agar. In the experiment on N-OH-Trp-P-1, 0.03 μg/plate of Trp-P-1 was incubated with 0.5 ml of S-9 mix for 7min at 37℃ to obtain the N-OH-Trp-P-1, and then the enzymes were completely inactivated in boiling water for 2 min.

Preparation of grilled beef extract(EGB). Sliced beef (fillet, purchased from a market) was grilled to medium doneness, and dried in vacuum. Five g of the grilled beef was miced and extracted with 20 ml of dimethyl sulfoxide (DMSO) for 60 min at room temperature. The solution(EGB) was sterilized with filtration (Minisart NML 0.45 μm, Sartorius). It contained 2.5 mg of residues /100 μl.

Preparation of wine condensates. The white wine and the red wine (Mercian Co. Ltd., Tokyo) were commercially obtained. After complete removal of alcohol from each wine under reduced pressure using a rotary vacuum evaporator, it was concentrated under the same condition. The white wine condensate contained 23.6 mg of residues /100 μl and red one 16.6 mg /100 μl. These solution were also sterilized with filtration.

Preparation of cigarette smoke condensate (CSC). Cigarette (Seven Star, Japan Tobacco Inc., Tokyo) was commercially obtained. The cigarette smoke was sacked into 50 ml of DMSO by an aspirator until the solution become colored deep brown. The solution (CSC) contained 1.6 mg of residues /100 μl and was sterilized with filtration.

Preparation of extracts of automobile exhaust. The soot of exhaust was collected from the muffler of each automobile by using spatula, and was ground into powder.

One g of powder of each exhaust was extracted with 50 ml of DMSO for 60 min at room temperature. Each solution was sterilized with filtration. Extracts of gasoline engine exhaust (EGE) contained 0.18 mg of residues /100 μl and diesel engine exhaust(EDE) 0.78 mg /100 μl.

Results and Discussion

Mutagenicities of some mutagenic substances.

Table 1 shows mutagenicities of some mutagenic substances used in this study with or without metabolic activation. The present author prepared crude beef extracts (EGB) as an example of mutagenic substances in daily foods. EGB showed dose dependent mutagenicity on *S. typhimurium* TA98 only in the presence of S-9 mix. The previous studies[10-12,33] showed that MeIQ, MeIQx, and other heterocyclic amines identified in broiled beef and fish were mutagenic. Therefore, the major mutagenic compounds contained in EGB may be heterocyclic amines and/or quinolines, which need metabolic activation to cause mutation for TA98. The present author also prepared two kinds of environmental carcinogenic substances, such as the cigarette smoke condensate and the extracts of automobile exhaust. The cigarette smoke condensate also showed the mutagenicity only in the presence of S-9 mix(Table 1). Many carcinogens, such as benzo-(a)-pyrene, benzanthoracene, nitrosoamine and so on, were detected in the cigarette smoke, and it was suggested that these compounds were mainly factors related to heavy smokers carcinogenesis through epidemiological investigations.[34-38] The automobile exhaust, especially diesel engine exhaust, is also important environmental factors related to human carcinogenesis. Some workers[39-42] have investigated the carcinogenicity of dinitropyrene, which was detected in the diesel engine exhaust particulates, as a strong carcinogen. In this study, the diesel engine exhaust extract(EDE) and gasoline one(EGE) were used. These extracts used showed dose-dependent mutagenicity on TA98 both with and without metabolic activation. TA98 was about 2-3 fold more sensitive to EDE without S-9 mix than with it. Conversely, it was about 2 fold more sensitive to EGE with S-9 mix than without it. No killing effect was observed, at least with all doses tested in this study (data not shown).

Effects of the wine condensates on the mutagenicity of EGB

The antimutagenic activities of two kinds of wine condensate were evaluated for TA98 using EGB. As shown in Table 2, the red wine condensate effectively decreased the mutagenicity of EGB in a dose-dependent manner down to the level of spontaneous

mutation with S-9 mix; values for the relative activity were 47% and 8% at 8.3 and

Table 1. Mutagenicities of Some Mutagenic Substances against *S.typhimurium* TA98[a]

Mutagenic substances	dose (mg)	His⁺revertants/plate[b]	
		+ (S−9mix)	− (S−9mix)
EGB (Extract of grilled beef)	0.22	195±13	8±5
	0.32	410±22	8±3
	0.54	595±35	18±3
	1.08	1037±186	15±5
CSC (Cigarette smoke condensate)	0.05	185±22	4±3
	0.13	285±26	0±2
	0.20	239±15	3±2
AF−2	0.02 (μg)	0±1	154±13
	0.04	3±2	234±21
	0.08	1±2	515±42
N−OH−Trp−P−1	0.03 (μg)	−	860±58
EDE (Exract of diesel engine exhaust)	0.08	145±12	461±26
	0.16	336±28	673±46
	0.23	397±31	1344±116
EGE (Exract of gasoline engine exhaust)	0.18	274±18	170±18
	0.27	369±32	221±15
	0.36	574±22	231±21

a : Mutagenicity was tested with or without S-9 mix.
b : Each value represents mean ±S.D. of triplicate plates. The values shown have had the spontaneous mutation frequency substrated.

16.6mg/plate, respectively. Conversely, the white wine condensate inhibited EGB-induced mutation by only 1 to 14% at the concentration of about being same.

Effects of the wine condensates on the mutagenicity of cigarette smoke condensate(CSC)

As shown in Table 3, the red wine condensate effectively decreased the mutagenicity of CSC in a dose-dependent manner down to the level of spontaneous mutation with S-

Table 2. Effect of the Wine on the Mutagenicity of Extract of Grilled Beef (EGB) against *S.typhimurium* TA98[a]

Mutagenic substance (mg/plate)	Wine condensate (mg/plate)		His$^+$revertants/plate[b] TA98	Relative activity (%)[c]
EGB 0.54			712±54	100
	+Red wine	1.70	761±68	107
		4.97	535±32	75
		8.30	338±42	47
		16.60	54±11	8
	+White Wine	2.40	705±54	99
		7.10	684±62	84
		11.82	627±48	88

a : Mutagenicity was tested with S-9 mix.
b : Each value represents mean ±S.D. of triplicate plates. The values shown have had the spontaneous mutation frequency substrated.
c : Numbers indicate the relative activity expressed as a percentage of His$^+$revertant colony counts per plate with the wine condensates to those without wine condensates.

9 mix; mutagenic activity of CSC decreased to 22 and 6% by 8.3 and 16.6 mg/plate of the red wine condensate, respectively. On the other hand, the white wine condensate showed no antimutagenic effect on the CSC-induced mutagenicity except for the addition of 11.2 mg/plate.

Effects of the wine condensates on the mutagenicity of AF-2

AF-2 needs no metabolic activation to cause mutation for TA98. The effects of wine condensates on the mutagenicity of AF-2 without metabolic activation by the enzymes were shown in Table 4. Both wine condensates showed no significant decrease

Table 3. Effect of the Wine on the Mutagenicity of Cigarette Smoke Condensate (CSC) against *S.typhimurium* TA98[a]

Mutagenic substance (mg/plate)	Wine condensate (mg/plate)		His$^+$revertants/plate[b] TA98	Relative activity (%)[c]
CSC 0.12			272±15	100
	+Red wine	1.70	295±22	108
		4.97	162±12	60
		8.30	59±6	22
		16.60	17±8	6
	+White Wine	2.40	298±32	110
		7.10	287±24	106
		11.82	182±23	67

a : Mutagenicity was tested with S-9 mix.
b : Each value represents mean ±S.D. of triplicate plates. The values shown have had the spontaneous mutation frequency substrated.
c : Numbers indicate the relative activity expressed as a percentage of His$^+$revertant colony counts per plate with the wine condensates to those without wine condensates.

of the mutagenicity of AF-2 without S-9 mix; mutagenic activity of AF-2 was only inhibited by 12 and 1% at concentrations of 8.3 mg/plate of the red wine condensate and 11.8 mg/plate of white, respectively.

Effects of the wine condensates on the mutagenicity of N-OH- Trp-P-1(activated Trp-P-1)

From all above results, it is suggested that one possible mechanism for the antimutagenic activity of the red wine condensate may be that they inhibit the

Table 4. Effect of the Wine on the Mutagenicity of AF-2 against *S.typhimurium* TA98[a]

Mutagenic substance (μg/plate)	Wine condensate (mg/plate)		His⁺revertants/plate[b] TA98	Relative activity(%)[c]
AF−2 0.04			275±18	100
	+Red wine	1.70	207±15	75
		4.97	248±21	90
		8.30	241±11	88
	+White Wine	2.40	206±12	75
		7.10	282±19	103
		11.82	272±16	99

a : Mutagenicity was tested without S-9 mix.
b : Each value represents mean ±S.D. of triplicate plates. The values shown have had the spontaneous mutation frequency substrated.
c : Numbers indicate the relative activity expressed as a percentage of His⁺revertant colony counts per platewith the wine condensates to those without wine condensates.

metabolic activation by the enzymes. Trp-P-1 is known to be converted into the N-hydroxy form, N-OH-Trp-P-1, by metabolic enzymes to develop mutagenic activity.[11,12] Then the present author tested the effects of the wine condensates on the mutagenicity of N-OH-Trp-P-1 prepared by incubation of Trp-P-1 with S-9 mix just before the mutation assay. N-OH-Trp-P-1 was prepared as described in Materials and Methods section. After inactivating the enzymes, the wine condensates were added to the mixture. As shown in Table 5, both of the wine condensates greatly decreased the mutagenicity of N-OH-Trp-P-1; the mutagenicity completely decreased to 6 and 8% with 8.3 mg/plate of the red wine condensate and 11.8 mg/plate of the white, respectively. These results suggest that the wine condensates may inhibit the mutagenic activity of this mutagen partly by direct reactions with activated mutagen rather than by the inactivation of metabolic enzymes, and they may interfere the reactions between N-OH-Trp-P-1 and DNA.

Effects of the wine condensates on the mutagenicities of the extracts of automobile exhaust (EDE and EGE).

As described previously, EDE and EGE showed dose dependent mutagenicity on S. *typhimurium* TA98 both with metabolic activation by enzymes and without. The effects

Table 5. Effect of the Wine on the Mutagenicity of N-OH-Trp-P-1 against *S.typhimurium* TA98[a]

Mutagenic substance (μg/plate)	Wine condensate (mg/plate)	His⁺revertants/plate[b] TA98	Relative activity(%)[c]
N−OH−Trp−P−1 0.03		801±64	100
	+Red wine 1.70	296±23	37
	4.97	112±9	14
	8.30	51±9	6
	+White Wine 2.40	447±39	56
	7.10	152±22	19
	11.82	68±11	8

a : Mutagenicity was tested without S-9 mix.
b : Each value represents mean ±S.D. of triplicate plates. The values shown have had the spontaneous mutation frequency substrated.
c : Numbers indicate the relative activity expressed as a percentage of His⁺revertant colony counts per plate with the wine condensates to those without wine condensates.

of the wine condensates on the mutagenicities of EDE and EGE with or without metabolic activation were shown in Table 6 and 7, respectively. In the presence of S-9 mix, both the wine condensates show the increase of EDE-induced mutation rather than decrease the mutation. The present author has investigated the effects of many foodstuffs on the EDE-induced mutation and could find no any effective foodstuff under the metabolic activation system. On the other hand, the red wine condensate shows the effective decrease of EGE-induced mutation; mutagenic activity was reduced to 12 percent by 16.6 mg/plate of the red wine condensates. In the absence of S-9 mix, the red wine condensates greatly decreased only the mutagenicity of EGE; the mutagenicity completely disappeared with 8.3 mg/plate of the red wine condensate.

Effects of the extract of grape species and skin or the extract of grape sarcocarp on the mutagenicity of CSC

In most above experiments, the red wine extract showed much stronger antimutagenic activity than the white wine. These results suggest that the red wine

Table 6. Effect of the Wine on the Mutagenicity of the Extract of Automobile Exhaust against *S.typhimurium* TA98 in the Presence of S-9 mix

Mutagenic substances (mg/plate)	Wine condensate (mg/plate)		His⁺revertants/plate[a] TA98	Relative activity(%)[b]
EDE 0.31			462±36	100
	+Red wine	1.70	687±59	149
		4.97	644±52	139
		8.30	720±46	156
	+White Wine	2.40	628±48	136
		7.10	558±45	121
		11.82	697±52	151
EGE 0.36			405±56	100
	+Red wine	1.70	326±22	80
		4.97	341±58	84
		8.30	303±41	75
		16.60	49±12	12

a : Each value represents mean ±S.D. of triplicate plates. The values shown have had the spontaneous mutation frequency substrated.
b : Numbers indicate the relative activity expressed as a percentage of His⁺revertant colony counts per plate with the wine condensates to those without wine condensates.

Table 7. Effect of the Wine on the Mutagenicity of the Extract of Automobile Exhaust against *S.typhimurium* TA98 in the Absence of S-9 mix

Mutagenic substances (mg/plate)	Wine condensate (mg/plate)		His⁺revertants/plate[a] TA98	Relative activity(%)[b]
EDE 0.16			584±45	100
	+Red wine	1.70	435±33	74
		4.97	553±62	95
		8.30	501±39	86
	+White Wine	2.40	503±48	86
		7.10	598±45	102
		11.82	621±52	106
EGE 0.36			235±18	100
	+Red wine	1.70	127±12	54
		4.97	62±14	26
		8.30	0±1	0

a : Each value represents mean ±S.D. of triplicate plates. The values shown have had the spontaneous mutation frequency substrated.
b : Numbers indicate the relative activity expressed as a percentage of His⁺revertant colony counts per plate with the wine condensates to those without wine condensates.

may contain more effective antimutagenic substances. It is well known that the red wine is made from whole fruit of grape with species and skin, and while the white wine is made from grape free of species and skin. Therefore, the red wine contains various chemical compounds which are from species and skin. For example, the polyphenol of the anthocyanidin is included for the red wine. For the plant, much kinds of polyphenol is included, and many workers have studied their physiological effects.[43-45] In the previous paper,[46] the present author also had studied the antimutagenic activity of chlorogenic acid and caffeic acid, which were polyphenol in caffee beans. Therefore, one of main antimutagenic substances in the red wine may be the anthocyanidin from species and skin of the grape. Then, in this experiment, the dimethyl sulfoxide extract of grape species and skin or the extract of grape sarcocarp were prepared, and each extract was examined for antimutagenicity on CSC. As shown in Table 8, the extract of species and skin significantly decreased the mutation induced by CSC much more than the extract of sarcocarp, in accord with expectations.

Table 8. Effect of the Extract of Grape Species and Skin or the Extract of Grape Sarcocarp on the Mutagenicity of CSC against *S.typhimurium* TA98[a]

Mutagenic substance (mg/plate)	Extract (mg/plate)		His$^+$revertants/plate[b] TA98	Relative activity(%)[c]
CSC 0.10			253±15	100
	+Species and skin	1.92	189±21	75
		5.77	112±12	44
		9.61	62±12	25
	+Sarcocarp	2.15	302±32	119
		6.45	270±31	107
		10.75	223±16	88

a : Mutagenicity was tested with S-9 mix.
b : Each value represents mean ±S.D. of triplicate plates. The values shown have had the spontaneous mutation frequency substrated.
c : Numbers indicate the relative activity expressed as a percentage of His$^+$ revertant colony counts per plate with the wine condensates to those without wine condensates.

It has been epidemiologically suggested that there are many environmental factors related to human carcinogenesis, but some foodstuffs may have preventive effectiveness on them. At present, the present author has no other date about the antimutagenic effects of the wine condensates. These findings in this study, however, seem to suggest that much attention should be paid to the red wine to clarify the antimutagenic activity of the wine condensate.

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