大韓獸醫學會誌(1992) 第32券 第4號
Korean J Vet Res (1992) 32(4) : 629~633

The effect of caffeine on promotion step of diethylnitrosamine-initiated hepatic altered foci in a mid-term induction system

Sung-ho Kim, Cha-soo Lee*
Korea Cancer Center Hospital, Korea Atomic Energy Research Institute
*College of Veterinary Medicine, Kyungpook National University**
(Received June 8, 1992)

Caffeine이 diethylnitrosame에 의해 유도되는 preneoplastic hepatic altered foci 형성의 promotion단계에 미치는 효과

김 성 호·이 차 수*
한국원자력연구소 원자력병원
경북대학교 수의과대학*
(1992년 6월 8일 접수)

초록 : Caffeine이 랫드의 간조직에서 diethylnitrosamine(200mg/kg B.W., DEN)에 의해 유도되는 preneoplastic altered foci형성의 promotion단계에 미치는 효과를 관찰한 바 다음과 같은 결과를 얻었다. Altered foci의 지표로 사용되는 glutathione S-transferase(GST-P)-positive foci의 수는 caffeine 음수 ㎖당 2mg 병행투여군(3.10±2.74) 및 1mg병행 투여군(5.86±2.83) 모두에서 DEN 단독투여 대조군(11.55±5.82)에 비하여 현저히 낮게 나타났으며 면적 또한 caffeine 2mg 병행투여군(0.13±0.11), 1mg 병행투여군(0.21±0.12)에서 DEN 단독투여 대조군(0.76±0.33)에 비하여 유의성있는 낮은 수치가 관찰되었다. 간 세포배양에서 unscheduled DNA synthesis(UDS)는 DEN(250 ㎍/㎖ of medium)단독처리군에 비하여 caffeine(200 ㎍/㎖ of medium) 처리시 약 70% 감소하였다. 이러한 결과는 caffeine이 간암발생의 promotion단계에 작용하여 억제효과를 나타냄을 암시하며 이는 DNA회복의 억제와 관계됨을 알 수 있었다.

Key words : caffeine, glutathione S-transferase placental form, liver altered foci, promotion step, unscheduled DNA synthesis.

Introduction

Chemically, caffeine (1, 3, 7-trimethylxanthine) is a plant alkaloid, structurally a deoxypurine which is related to uric acid and to two purine DNA base pairs, adenine and guanine. The alkaloid does have a propound effect on a number of physiological and biochemical processes in mammalian tissues. Caffeine has been reported to be stimulatory to the central nervous system, heart and skeletal muscles, pulmonary blood and air supply, gastric juice secretion, urine secrition, whereas it inhibits stomach smooth muscle peristalsis and fertility. Caffeine has also been reported to increase Ca^{++} ion availability, blood glucose and fatty acid concentrations and tissue cAMP levels, the latter activity mediated via the inhibitory effect of alkaloid on 3', 5'-phosphodiesterase activity.[1-3]

Caffeine has been reported to influence neoplastic pro-

cesses in experimental animals. In the preceding communication, we examined the effect of caffeine consumption on the development of DEN-induced hepatic altered foci in the initiation studies.[4] In this report we further examined the relationship between caffeine consumption and the development of DEN-induced hepatic altered foci in the promotion step.

Materials and Methods

Animals : Six-week-old male Sprague Dawley rats of our institute colony were housed in polycarbonate cages. They were fed on a standard animal diet (NIH-7-open formula ration) and given tap water ad ribitum.

Chemicals : Caffeine was obtained from Sigma. DEN was from Wako Pure Chemical Co., Ltd., Japan and rabbit anti GST-P, used in immunohistochemical studies, was kindly provided by Prof. Kiyomi Sato, Medical school of Hirosaki University, Japan. Affinity-purified biotin-labeled goat anti-rabbit immunoglobulin G and avidin-biotin-peroxidase complex (Vectastain ABC Kit, PK 4001, ABC) were obtained from Vector Laboratories Inc.

Experimental design : The experimental schedule followed was shown in fig 1. A total of 70 rats were divided into 4 groups. Group 1, 2 and 3 were treated with a single intraperitoneal injection of DEN at 200mg/5mℓ of saline/kg B. W. Two weeks later after DEN or saline injection, the rats of group 1, group 2, and group 4 were given drinking water containing caffeine at the concentration of 0.1% or 0.2% for 6 weeks. Three weeks after the DEN or solvent injection, two-thirds partial hepatectomy was performed on all animals. The animals were sacrificed under ether anesthesia at week 8 after DEN or solvent injection. All animals were fasted 12 hours prior to sacrifice.

Histological examination and quantitation of GST-P$^+$ foci : The livers were excised and cut into 2~3mm thick sections with a razor blade. Three slices, one from the right posterior and two from the right anterior lobes, were fixed in ice-cold acetone and processed for embedding in paraffin and subsequent immunohistochemical examination of GST-P$^+$ foci by the ABC method decribed previously[5,6] and hematoxylin and eosin (HE) staining. The numbers/cm^2 and areas (mm^2/cm^2) of GST-P$^+$ foci of over 0.2mm in diameter were measured using a color video image processor (Kontron Ltd, Germany). The location of GST-P positive site and HE staining lesions were examined in successive serial sections.

Primary monolayer cultures and DNA repair test : Liver cells were prepared by the collagenase perfusion technique according to Butterworth.[7] Cell viability was assessed by the trypan blue dye exclusion test. Cell cultures were established by inoculation of 3×10^5/viable cells into 50-mm plastic Petri dishes containing 3mℓ of Williams medium E supplemented with 10% fetal bovine serum and gentamycin(50 μg/mℓ). The cells were distributed over the cover slip by shaking. After an attechment period of 90 minutes, the medium was removed and replaced by 3mℓ of medium, and the monolayer cultures were incubated for 20 hours in water-saturated 5% CO_2 : 95% air incubator, with DEN (250 μg/mℓ), [^{3}H] thymidine (10 μCi/mℓ, 81.9Ci/mmol, NEW) and with or without caffeine (200 μg/mℓ). The cells were washed three times with cold Hank's balanced solt solution and treated with 1% sodium citrate solution for 10 minutes. Then the cells were fixed in acetic acid : ethanol (1 : 3) for three 10-minutes changes and cover slips were removed from the wells and placed cell-side-up to dry. Dried cover slips were mounted cell-side-up on microscopic slides with permount, and dipped in Kodak NTB-2 emulsion for autoradiography, and exposed for 12 days at -20℃. Autoradiographic grains were then developed and stained Wright's solution. Autoradiographs were counted in 50 consecutive cells of each slide. The highest cytoplasmic background count for each cell was subtracted from the nuclear count to obtain the net nuclear grains due to DNA repair synthesis.

Statistical analysis were performed Student's t-test.

Results

The body an liver weights showed no difference between groups. But the liver weights of animals treated with caffeine were fewer than that of animals in group 3 (Table 1). Grossly, livers were smooth and no cirrhotic changes are seen. Microscopically, the altered focus were demarcated from the surrounding parenchimal tissue but it was difficult to distinguish focus from surrounding hepatocyte in the HE stained tissue. Areas of the focus showed strong activity of GST-P(Fig 2).

Table 1. Mean body and liver weight

Group	No. of rat	Body weight(g) (mean±S.D.)	Liver weight(mean±S.D.)	
			g	mg/g body weight
1. DEN →caffeine(2mg)	20	341.8±20.4	12.5±1.5[a]	36.7±4.1
2. DEN →caffeine(1mg)	18	366.2±27.6	14.1±1.5	38.5±3.4
3. DEN →BD	15	375.5±42.5	14.1±1.7	38.0±6.0
4. Saline →caffeine(2mg)	14	347.3±19.7	13.1±1.7	38.0±6.0

DEN : diethylnitrosamine, BD : basal diet.
[a] $P<0.01$ as compared with group 3.

Table 2. Quantitative value of GST-P positive foci in the liver

Group	No. of rat	GST-P positive foci(mean±S.D.)		
		Number/cm^2	Area(mm^2)/cm^2	Maximum diameter(mm)
1. DEN→ →caffeine(2mg)	20	3.10±2.74[a]	0.13±0.11[a]	0.33±0.06[a]
2. DEN →caffeine(1mg)	18	5.86±2.83[b]	0.21±0.12[a]	0.31±0.05[a]
3. DEN →BD	15	11.55±5.82	0.76±0.33	0.40±0.07
4. Saline →caffeine(2mg)	14	–	–	–

DEN : diethylnitrosamine, BD : basal diet. [b] $P<0.05$ as compared with group 3
[a] $P<0.005$ as compared with group 3.

Table 3. The effect of caffeine on unscheduled DNA synthesis(UDS) in primary hepatocyte culture

Group	UDS(grains/nucleus)
Control	1.61±2.66[a]
Caffeine 200㎍	
DEN 250㎍	2.06±1.95
DEN 250㎍+caffeine 200㎍ (simultaneous treatment)	15.2±5.93[b]
Caffeine 200㎍+DEN 250㎍ (4hrs before DEN)	5.95±2.72
DEN 250㎍+caffeine 200㎍ (4hrs after DEN)	5.9±2.67
	7.45±2.33

[a] Mean±S.D.
[b] $P<0.005$ as compared with other groups.

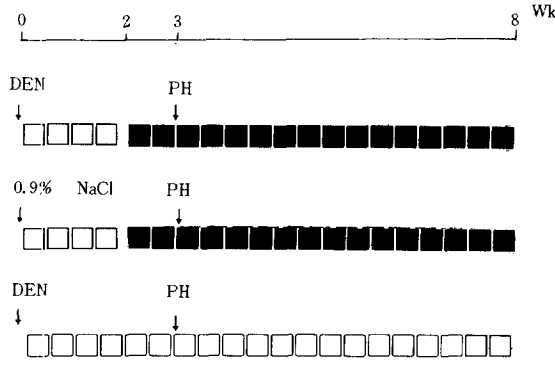


Fig 1. Design of experiment.
↓ : i.p. injection of 200 mg of DEN/kg body weight or 0.9% NaCl solution, ↓ PH : partial hepatectomy, □ : basal diet, ■ : caffeine 1 mg or 2 mg/㎖ of drinking water.

The average total numbers, total areas of foci per cm^2 and maximum diameter of liver tissue in the each group are shown in Table 2. Treatment of caffeine (group 1, group 2) resulted in a significant decrease in both the number ($p < 0.05$), area ($p < 0.005$) and maxinun diameter ($p<0.005$) of GST-P$^+$ foci compared with those in group 3. Caffeine alone could not induced the GST-P$^+$ foci.

The results obtained from the autoradiographic evaluation of UDS are reported in table 3. In autoradiographs of hepatocyte primary cultures(HPC) treated with DEN alone, the average net nuclear grain count was 15.2±5.93. Statistically significant decrease in the average

number of grains per nucleus was observed in HPC treated with DEN and caffeine(Fig 3, 4).

Discussion

We investigated the effect of caffeine on the promotion step of DEN-induced rat preneoplastic hepatic altered foci.

In the promotion step, direct interaction between DEN and caffeine and altered metabolism of DEN by caffeine are ruled out, because DEN is short acting and DEN was injected 2 weeks before caffeine treatment. One possible explanation is that cyclic adenosine 3', 5' -monophosphate (cAMP) promotes cell differentiation[8], resulting in decrease of tumors, because caffeine is known to increase cellular level of cAMP by inhibiting phosphodiesterase.[8~10] However, contribution of cAMP for inhibiting teratogenesis and carcinogenesis will be ruled out, because yields of urethan-induced tumors and malformations were not reduced by theophylline which elevates cellular level of cAMP more effectively than does caffeine.[9~11] The other possible mechanism is that caffeine bind to damaged DNA, inhibits DNA synthesis,[12] interferes with the elongation of DNA.[8] In this study, we confirmed that the effects of caffeine were attributed to inhibition of unscheduled DNA synthesis.

Since a good correlation exists between ability to inhibit in the mid-term test and in long-term experiments,[13,14] it might be expected that caffeine also exert long-term inhibitory potential.

Conclusion

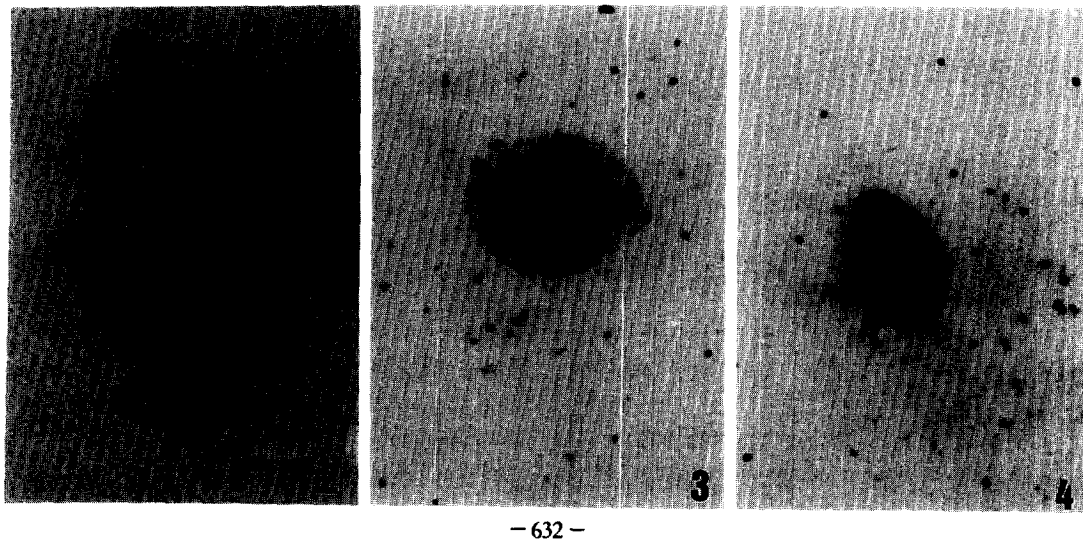
The modification potentials of caffeine on the development of preneoplastic hepatic enzyme altered foci were examined in an *in vivo* mid-term assay system. The number and area of glutathione S-transferase placental form (GST-P) positive foci of the liver was significantly reduced in rats given DEN (200mg/kg B.W. I.P.) followed by caffeine (2mg or 1mg/mℓ of drinking water) as compared with the controls given carcinogen alone. Unscheduled DNA synthesis(UDS) decreased approximately 70% in the hepatocyte treated with caffeine. These results suggested that the antipromotive effect of caffeine might be associated with the suppression of DNA repair.

Legends for figures

Fig 2. A preneoplastic altered focus induced with DEN in hepatectomized rat. Positive immunohistochemical reaction of GST-P. X 20.

Fig 3. Autoradiograph of primary cultured hepatocyte. The cell was incubated with DEN and [^{3}H]thymidine stained with Wright's solution after 12 days exposure to the emulsion. The fine grains on the nucleus represent unscheduled DNA synthesis. X 500.

Fig 4. Autoradiograph of primary cultured hepatocyte. The cell was incubated with DEN, [^{3}H]thymidine and caffeine and stained with Wright's solution after 12 days exposure to the emulsion. The cell contains less grains than the cells of Fig 3. X 500.



References

1. Pozniak PC. The carcinogenicity of caffeine and coffee : A review. *J Am Diet Assoc* 1985 ; 85 : 1127~1133.
2. Stavric B. Methylxanthines : toxicity to humans. 2. Caffeine. *Fd Chem Toxic* 1988 ; 26 : 645~662.
3. Booth NH. Drug acting on the central nervous system. In : Booth NH and McDonald LE, ed. *Veterinary pharmacology and thrapeutics*. 5th ed. Iowa State Univ Press. 1982 ; 350~351.
4. Kim SH,Lee CS. The effect of caffeine on initiation step of diethylnitrosamine-initiated hepatic altered foci in a mid-term induction system. *Korean J Vet Res* 1991 ; 31 : 337~342.
5. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex(ABC) in immunoperoxidase techniques : a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 1981 ; 29 : 577~580.
6. Jang JJ, Cho KJ, Kim SH. Effects of allyl sulfide, germanium and NaCl on the development of glutathione S-transferase p-positive rat hepatic foci initiated by diethylnitrosamine. *Anticancer Res* 1989 ; 9 : 273~276.
7. Butterworth BE, Ashby J, Bermudez E, et al. A protocol and gulde for the in vitro rat hepatocyte DNA-repair assay. *Mutat Res* 1987 ; 189 : 113~121.
8. Butcher RW, Sutherland EW. Adenosine 3', 5' -phosphate in biological materials. *J Biol Chem* 1962 ; 237 : 1244~1250.
9. Amer MS, Kreiglbaum WE. Cyclic nucleotide phosphodiesterases : properties, activators, inhibitors, structure-activity relationships, and possible role in drug development. *J Pharm Sci* 1975 ; 64 : 1~37.
10. Beavo JA, Rogers NL, Crofford OB, et al. Effects of xanthine derivatives on lipolysis and on adenosine 3', 5' -monophosphate phosphodiesterase activity. *Mol Pharmacol* 1970 ; 6 : 597~603.
11. Nomura T. Comparative inhibiting effects of methylxanthines on urethan-induced tumors, malformations, and presumed somatic mutations in mice. *Cancer Res* 1983 ; 43 : 1342~1346.
12. Cleaver JE. Repair processes for photochemical damage in mammalian cells. *Advan Radiat Biol* 1974 ; 4 : 1~74.
13. Ito N, Tsuda H, Tatematsu M, et al. Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione S-transferase placental form positive foci in rats-an approach for a new medium-term bioassay system. *Carcinogenesis* 1988 ; 9 : 387~394.
14. Ogiso T, Tatematsu M, Tamano S, et al. Comparative effects of carcinogens on the induction of placental glutathione S-transferase-positive liver nodules in a short-term assay and of hepatocellular carcinomas in a long-term assay. *Toxicol Pathol* 1985 ; 13 : 257~265.