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Promotion Effect of a Choline-deficient L-amino Acid-defined (CDAA) Diet on Hepatocellular Foci Formation Initiated by Diethylnitrosamine in Fischer 344 Rats

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Abstract: The purpose of this study is to determine which combination of choline-deficient L-amino acid-defined (CDAA) diet as an endogenous factor, phenobarbital (PB) as an exogenous factor, and partial hepatectomy (PH), has the most promotive potential on hepatocellular foci formation initiated by diethylnitrosamine (DEN) in Fischer 344 rats. Experimental groups were treated with various combinations of DEN administration, PB administration, PH and CDAA diet. Formation of hepatocellular foci after eight weeks was examined by glutathione S-transferase placental form (GST-P) immunological staining. The focal area was markedly elevated in the CDAA+DEN group in comparison with the CDAA-only group or the CRF-1(basal diet)+DEN group. CDAA diet was shown to have a strong promotion effect on initiated cells by DEN. In the CRF-1+DEN-treated animals, the additional PB and PH treatment elevated the number of small foci, but slightly did the focal area. The effects of the additional PB and PH treatment in the CDAA+DEN-treated animals were not clear as they were masked by the strong promotion effect of CDAA diet. These results showed that CDAA diet was more promotive than the PB and PH treatment in inducing hepatocellular foci formation initiated by DEN in rats, and the additional PB and PH treatment could not be promotive furthermore in the CDAA+DEN-treated animals. (*J Toxicol Pathol* 2001; **14**: 105–112)

Key words: choline-deficient L-amino acid-defined diet, diethylnitrosamine, phenobarbital, partial hepatectomy, glutathione S-transferase placental form, hepatocellular foci

Introduction

Hepatocarcinogenesis is a multistage process, the stages of initiation, promotion, and progression, and liver tumor is predicted to be induced after a long period of preneoplastic lesions^{1,2}. In vivo testing for prediction of hepatocarcinogenic potential of test substance in human using rodents takes a long-term. Medium-term hepatocarcinogenesis bioassay systems in rats employ an initiator such as diethylnitrosamine (DEN), followed by several weeks of exposure of test substance^{3,4}. These systems employ hepatocellular enzyme-altered foci caused by test substance as the endpoint marker lesion^{3,5–7}. Hepatocellular enzyme-altered foci expresses multiple phenotype and is detectable using immunological staining

by γ -glutamyltranspeptidase (γ -GTP), glutathione S-transferase placental form (GST-P) and others^{8–11}. Partial hepatectomy (PH) is carried out to enhance these initiators or test substance's effects^{12–15}. It has been reported that phenobarbital (PB), a well-known promoter^{16–19}, was not carcinogenic but it caused preneoplastic lesion in a two-year feeding study in Fischer 344 rats²⁰.

Carcinogen-treated rats fed a choline-deficient (CD) diet induces a preneoplastic lesion²¹. Meanwhile, choline-deficient L-amino acid-defined (CDAA) diet was developed by replacing the proteins by pure amino acids to give an equivalent amino acid profile. In constant with semipurified CD diet, CDAA diet has no contamination of carcinogens or choline. Therefore, it is thought that CDAA diet has a stronger effect to induce liver tumor than semipurified CD diet in rats^{22–24}, and the effect of CDAA diet is caused entirely by an endogenous mechanism.

The purpose of this study is to determine which combination of CDAA diet as an endogenous factor, PB as an exogenous factor, and PH, has the most promotive potential on hepatocellular foci formation initiated by DEN in Fischer 344 rats.

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Materials and Methods

Compounds

Diethylnitrosamine (DEN, Sigma Chemical Co., USA) and phenobarbital (PB, Wako Pure Chemical Industries Ltd., Osaka) were used as an initiator and promoter, respectively. Saline (Otsuka Pharmaceutical Factory, Inc., Tokushima) was used as vehicle for both compounds.

Animals

The eighty six male 4-week-old Fischer 344 rats used in this study were obtained from Charles River Japan, Inc.(Kanagawa). Grouping of the rats was performed according to the latest body weight. The animals were housed, two/cage in a climate-controlled animal room at temperature of $24 \pm 3^\circ\text{C}$, relative humidity of $55 \pm 15\%$, and ventilation rate of 10–20 times/h with 12-hour illumination, and allowed free access to the prescribed diets in every group and tap water.

Groups and treatment methods

Experimental design is shown in Fig. 1. To the animals in group 1, only a CRF-1 basal diet (Oriental Yest Co., Ltd., Tokyo) was fed. To the ones in group 2, only a CDAA (Dyets Inc., USA) diet was fed. The CDAA diet, whose composition is shown in Table 1, was fed from the day before the 1st administration day of DEN (day 1) and was stored under refrigeration until use. To the ones in group 3, a CRF-1 basal diet was fed and DEN was treated. The dose of DEN was set at 95 mg/kg (2 ml/kg) and rats were intraperitoneally treated once a week for the first three weeks (day 1, 8 and 15). To the ones in group 4, a CDAA diet was fed and DEN was treated. To the ones in group 5, a CRF-1 basal diet was fed and given DEN and PH. In the partial hepatectomy (PH), the left lobe and the right and left sublobes of the median lobe of the liver were excised. PH was carried out on the day before the first administration of PB (day 28). To the ones in group 6, a CRF-1 basal diet was fed and given DEN, PH, and PB. The dose of PB was set at 60 mg/kg (2 ml/kg) and rats were intraperitoneally treated three times a week from week 5 (day 29, 32, 34, 36, 39, 41, 43, 46, 48, 50, 53, and 55). To the ones in group 7, a CDAA diet was fed and given DEN and PH. To the ones in group 8, a CDAA diet was fed and given DEN and PB. To the ones in group 9, a CDAA diet was fed and given DEN, PH, and PB. This experiment was started on 10 April 1997.

Blood chemistry examination

At autopsy (day 60–63), blood was collected from the ventral aorta and alkaline phosphatase (ALP, p-NPP substrate method)^{25,26}, glutamic-oxaloacetic transaminase (GOT, Henry altered method)²⁷, glutamic-pyruvic transaminase (GPT, Henry altered method)²⁷, γ -glutamyl transpeptidase (γ -GTP, γ -Glu-pNA substrate method)²⁸, total cholesterol (TCH, CHE-COD-POD method)²⁹, triglyceride (TG, LPL-GK-GPO-POD method)³⁰, and free fatty acid (FFA, ACS-MK-PK-LDH method)³¹ in serum measured

Fig. 1. Experimental design. CDAA was fed from the day before the 1st administration day of DEN (day 1). DEN was treated at day 1, 8, and 15. Partial hepatectomy (PH) was carried out at day 28. Phenobarbital (PB) was treated at day 29, 32, 34, 36, 39, 41, 43, 46, 48, 50, 53, and 55. Autopsy was carried out at day 60–63.

using an autoanalyzer (AU550, Olympus Optical Co., Ltd., Tokyo).

Pathological examination

At eight weeks (day 60–63), autopsy was performed after the animals were sacrificed by exsanguination under ether anesthesia. After the liver had been excised and fixed in 10% neutral buffered formalin fixative, paraffin sections and H.E.-stained specimens were prepared and microscopic examination was carried out.

Immunohistochemical staining using polyclonal antibodies against GST-P was applied to facilitate quantitative evaluation of altered hepatocellular foci. Paraffin sections were stained immunohistochemically using the labeled streptavidin-biotin-peroxidase complex (LSAB)

Table 1. Compositions of Choline-deficient L-amino acid-defined (CDAA) Diet

Ingredient	(g/kg diet)
L-Arginine	12.7
L-Histidine	3.4
L-Lysine-HCl	9.1
L-Tyrosine	5.7
L-Tryptophan	1.8
L-Phenylalanine	7.3
L-Methionine	1.7
L-Cystine	3.7
L-Threonine	4.6
L-Leucine	10.5
L-Isoleucine	6.1
L-Valine	6.3
Glycine	6.2
L-Proline	7.6
L-Glutamic acid	28.9
L-Alanine	5.1
L-Aspartic acid	15.8
L-Serine	7.2
Corn starch	100
Dextrin	100
Cellulose	50
Sucrose	406.67
Sodium bicarbonate	4.3
Corn oil	50
Primex	100
Modified AIN-76 salt mix	35
AIN-76A vitamin mix	10
Ferric citrate	0.33

method for binding of rabbit anti-rat GST-Yp polyclonal antibody (1 : 1000 ; BIO PREP, IRELAND). The analytical procedure for the GST-P-stained specimens was as follows. Photographs ($\times 10$) of GST-P-stained specimens of whole cross-section of liver were taken. Photographs were jointed to construct complete cross-sectional image. GST-P-positive foci (hereinafter foci) were painted out with felt pen. Cross-sectional images were processed by Image Scanner (GT-6500, SEIKO EPSON Co., Ltd., Nagano) (using EpScan Mac). The area of each focus and total cross-sectional area of each image were measured using Adobe Photoshop and NIH image. Number of foci per unit area and focal area per unit area were calculated from data on number of foci, mean focal area, total focal area, and total organ area.

Statistical evaluation

All examination data were statistically evaluated by F-t test. First, equal dispersion was tested by F test (significance level 5%). Student-t test was carried out where population variance was judged to be equal, and Welch-t test where it was judged different. Bilateral significance level was set at 5% and 1%.

Results

Survival, final body weight, and liver weight

Survival, final body weight, and liver weight are shown in Table 2. In the CDAA+DEN-treated groups (7 and 9), four animals died after PH. Final body weight was reduced in the CDAA-only group (2) in comparison with the CRF-1-only group (1), and was further reduced in the CDAA+DEN-treated groups (4, 7, 8, and 9) in comparison with the CDAA-only group (2). Liver weight was markedly elevated in the CDAA-only group (2) in comparison with the CRF-1-only group (1), and was further elevated in the CDAA+DEN-treated, but not PH-treated groups (4 and 8) in comparison with the CDAA-only group (2).

Autopsy and histopathological findings in liver, and blood chemistry examination

In the CDAA-treated groups (2, 4, 7, 8 and 9), a pale enlarged liver with a rough surface was observed in almost all animals. Histopathological findings of H.E.-stained specimens are shown in Table 3. In group 1 (CRF-1), no marked change was observed. Findings in group 2 (CDAA) not observed in group 1 (CRF-1) were fibrosis, severe vacuolar degeneration, oval cell formation, and altered hepatocellular foci. Findings in group 3 (CRF-1+DEN) not observed in group 1 (CRF-1) were slight vacuolar degeneration and altered hepatocellular foci. In group 4 (CDAA+DEN), fibrosis was more marked and oval cells more numerous than in group 2 (CDAA). The altered hepatocellular foci appeared pseudocircular separated by fiber. Almost all animals showed such changes to a severe degree. In group 5 (CRF-1+DEN+PH), altered hepatocellular foci were more marked than in group 3 (CRF-1+DEN). Group 6 (CRF-1+DEN+PH+PB) showed almost the same findings as group 3 (CRF-1+DEN). In group 7 (CDAA+DEN+PH), oval cells were slightly more numerous than in group 4 (CDAA+DEN). In groups 8 (CDAA+DEN+PB) and 9 (CDAA+DEN+PH+PB), findings were almost the same as in group 4 (CDAA+DEN).

In blood chemistry examination, in the CDAA-treated groups (2, 4, 7, 8, and 9), GOT, GPT, γ -GTP, and FFA were elevated and TG reduced in comparison with group 1 (CRF-1). In the CDAA+DEN-treated groups (4, 7, 8, and 9), γ -GTP was markedly elevated in comparison with group 2 (CDAA).

Analysis of GST-P-stained specimens

Figures for focal area are shown in Table 4, and for number of foci by size in Table 5. In group 1 (CRF-1), no foci were observed. In groups 2 (CDAA) and 3 (CRF-1+DEN), foci were observed (Fig. 2a, b). In group 4 (CDAA+DEN), in comparison with group 2 (CDAA), number of foci and focal area were elevated, as did number of foci in all size groups (Fig. 2e). In group 5 (CRF-1+DEN+PH), in comparison with group 3 (CRF-1+DEN), focal areas were elevated, as did number of foci of size 0.03–1.0 mm² (Fig. 2c). In group 6 (CRF-1+DEN+PH+PB), in

Table 2. Body Weight and Liver Weight

Group	DEN	CDAA	PH	PB	Number of animals		Body weight (day 57)			Liver weight		
					at Start	at Autopsy	(g)			(g)		
1	-	-	-	-	10	10	281.97 ± 14.09 CT			6.856 ± 0.440 CT		
2	-	+	-	-	10	10	222.86 ± 9.21 ** CT			11.689 ± 1.183 ** CT		
3	+	-	-	-	10	10	257.52 ± 5.11 ** CT			6.387 ± 0.226 ** CT		
4	+	+	-	-	10	10	192.65 ± 7.62 ** ** CT			13.430 ± 1.367 ** ** CT		
5	+	-	+	-	4	4	263.85 ± 3.96 ** *			6.335 ± 0.234 * -		
6	+	-	+	+	4	4	247.75 ± 4.10 ** **			6.225 ± 0.153 * -		
7	+	+	+	-	14	10#	169.28 ± 14.23 ** **			9.449 ± 2.352 * **		
8	+	+	-	+	10	10	190.32 ± 7.21 ** -			14.118 ± 1.370 ** -		
9	+	+	+	+	14	10#	175.32 ± 12.33 ** **			10.365 ± 1.011 * **		

DEN: diethylnitrosamine, CDAA: choline deficient L-amino acid defiend diet, PH: partial hepatectomy, PB: phenobarbital, CDAA (-): CRF-1 was fed to animals.

#: Differences from the start means number of the dead after PH.

, *: Significantly different from control (CT) (: p<0.01, *: p<0.05), -: Not signficantly different.

Table 3. Histopathological Findings of Liver

	Group	1	2	3	4	5	6	7	8	9
	DEN	-	-	+	+	+	+	+	+	+
	CDAA	-	+	-	+	-	-	+	+	+
	PH	-	-	-	-	+	+	+	-	+
	PB	-	-	-	-	-	+	-	+	+
	n	10	10	10	10	4	4	10	10	10
Findings	Grade	± + 2+ 3+	± + 2+ 3+	± + 2+ 3+	± + 2+ 3+	± + 2+ 3+	± + 2+ 3+	± + 2+ 3+	± + 2+ 3+	± + 2+ 3+
Fibrosis			5 5		1 9			9 1	1 9	10
Vacuolar degeneration			10 10		10 4	4		1 9	10	10
Ovar cell		4 6			10			7 3	10	10
Altered hepatocellular foci		7 3	2 8		2 8 4		3 1	10	1 9	10

DEN: diethylnitrosamine, CDAA: choline-deficient L-amino acid-defined diet, PH: partial hepatectomy, PB: phenobarbital, CDAA (-): CRF-1 was fed to animals.

Table 4. Number and Area of GST-P Positive Foci

Group	DEN	CDAA	PH	PB	n	Number of foci (/mm ² Liver)	Focal area (mm ² /mm ² Liver)
1	-	-	-	-	10	0.000 ± 0.000	0.000 ± 0.000
2	-	+	-	-	10	0.035 ± 0.014 CT	0.013 ± 0.011 CT
3	+	-	-	-	10	0.350 ± 0.083 CT	0.006 ± 0.002 CT
4	+	+	-	-	10	0.332 ± 0.179 ** CT	0.628 ± 0.112 ** CT
5	+	-	+	-	4	0.467 ± 0.162 - CT	0.015 ± 0.006 * CT
6	+	-	+	+	4	0.640 ± 0.103 ** -	0.017 ± 0.005 * -
7	+	+	+	-	10	0.231 ± 0.191 * - CT	0.681 ± 0.148 ** - CT
8	+	+	-	+	10	0.343 ± 0.202 ** - CT	0.640 ± 0.121 ** - CT
9	+	+	+	+	10	0.278 ± 0.141 ** - - -	0.656 ± 0.094 ** - - -

DEN: diethylnitrosamine, CDAA: choline deficient L-amino acid defined diet, PH: partial hepatectomy, PB: phenobarbital, CDAA (-): CRF-1 was fed to animals.

, *: Significantly different from control (CT) (: p<0.01, *: p<0.05), -: Not significantly different.

Table 5. Number of Foci (/mm² Liver) by Size

Group	DEN	CDA	PH	PB	n	Focal size 0–0.03 (mm ²)	Focal size 0.03–0.1 (mm ²)	Focal size 0.1–0.3 (mm ²)
1	–	–	–	–	10	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
2	–	+	–	–	10	0.003 ± 0.003 CT	0.008 ± 0.006 CT	0.011 ± 0.009 CT
3	+	–	–	–	10	0.312 ± 0.070 CT	0.035 ± 0.021 CT	0.003 ± 0.005 CT
4	+	+	–	–	10	0.082 ± 0.046 ** CT	0.079 ± 0.049 ** CT	0.065 ± 0.030 ** CT
5	+	–	+	–	4	0.335 ± 0.124 - CT	0.105 ± 0.030 ** CT	0.023 ± 0.014 - CT
6	+	–	+	+	4	0.515 ± 0.088 ** -	0.098 ± 0.018 ** -	0.022 ± 0.004 ** -
7	+	+	+	–	10	0.040 ± 0.026 ** * CT	0.045 ± 0.036 * - CT	0.045 ± 0.046 * - CT
8	+	+	–	+	10	0.110 ± 0.065 ** - CT	0.072 ± 0.041 ** - CT	0.064 ± 0.039 ** - CT
9	+	+	+	+	10	0.075 ± 0.034 ** - * -	0.047 ± 0.028 ** - - -	0.050 ± 0.025 ** - - -

Group	DEN	CDA	PH	PB	n	Focal size 0.3–1.0 (mm ²)	Focal size 1.0–3.0 (mm ²)	Focal size 3.0–10.0 (mm ²)
1	–	–	–	–	10	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
2	–	+	–	–	10	0.012 ± 0.005 CT	0.002 ± 0.003 CT	0.000 ± 0.001 CT
3	+	–	–	–	10	0.000 ± 0.000 CT	0.000 ± 0.000 CT	0.000 ± 0.000 CT
4	+	+	–	–	10	0.054 ± 0.036 ** CT	0.030 ± 0.022 ** CT	0.012 ± 0.007 ** CT
5	+	–	+	–	4	0.003 ± 0.002 ** CT	0.000 ± 0.000 - CT	0.000 ± 0.000 - CT
6	+	–	+	+	4	0.006 ± 0.006 ** -	0.000 ± 0.000 - -	0.000 ± 0.000 - -
7	+	+	+	–	10	0.046 ± 0.049 - - CT	0.030 ± 0.033 * - CT	0.012 ± 0.013 * - CT
8	+	+	–	+	10	0.047 ± 0.036 * - CT	0.026 ± 0.017 ** - CT	0.014 ± 0.011 ** - CT
9	+	+	+	+	10	0.043 ± 0.029 ** - - -	0.031 ± 0.028 ** - - -	0.018 ± 0.012 ** - - -

Group	DEN	CDA	PH	PB	n	Focal size 10.0–30.0 (mm ²)	Focal size 30.0–100.0 (mm ²)	Focal size 100.0– (mm ²)
1	–	–	–	–	10	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
2	–	+	–	–	10	0.000 ± 0.000 CT	0.000 ± 0.000 CT	0.000 ± 0.000 CT
3	+	–	–	–	10	0.000 ± 0.000 CT	0.000 ± 0.000 CT	0.000 ± 0.000 CT
4	+	+	–	–	10	0.008 ± 0.005 ** CT	0.002 ± 0.002 ** CT	0.002 ± 0.002 ** CT
5	+	–	+	–	4	0.000 ± 0.000 - CT	0.000 ± 0.000 - CT	0.000 ± 0.000 - CT
6	+	–	+	+	4	0.000 ± 0.000 - -	0.000 ± 0.000 - -	0.000 ± 0.000 - -
7	+	+	+	–	10	0.009 ± 0.008 ** - CT	0.002 ± 0.002 ** - CT	0.001 ± 0.002 ** - CT
8	+	+	–	+	10	0.006 ± 0.004 ** - CT	0.003 ± 0.002 ** - CT	0.002 ± 0.002 ** - CT
9	+	+	+	+	10	0.010 ± 0.009 ** - - -	0.004 ± 0.004 ** - - -	0.001 ± 0.001 ** - - -

DEN: diethylnitrosamine, CDA: choline deficient L-amino acid defined diet, PH: partial hepatectomy, PB: phenobarbital, CDA (-): CRF-1 was fed to animals. **, *: Significantly different from control (CT) (**: p<0.01, *: p<0.05), -: Not significantly different.

comparison with group 3 (CRF-1+DEN), number of foci and focal area were elevated, as did number of foci of size 0–1.0 mm² (Fig. 2d). In group 7 (CDA+DEN+PH), in comparison with group 4 (CDA+DEN), number of foci of size 0–0.03 mm² was reduced (Fig. 2f). In groups 8 (CDA+DEN+PB) and 9 (CDA+DEN+PH+PB), in comparison with group 4 (CDA+DEN), no change was observed (Fig. 2g, h).

Discussion

Focal area was markedly elevated in the CDA+DEN-treated group (4) in comparison with the CDA-only group (2) or the CRF-1+DEN group (3), but was not further elevated in the groups treated with PH, PB or PH+PB in

addition to CDA+DEN (7, 8, and 9). PB is a well-known exogenous promoter^{16–19}. The regeneration of liver after PH proceeds through compensatory hypertrophy of liver lobes^{32,33} and PH prompts synthesis of DNA in hepatocytes and cell division^{12–15}. But the effect of additional PH and PB treatment was not clear in the CDA+DEN-treated groups (4, 7, 8, and 9). These findings were taken as showing that CDA diet had a strong promotion effect in cells initiated by DEN. In the CDA+DEN-treated groups (4, 7, 8, and 9), histopathological examination showed marked fibrosis, and blood chemistry examination showed marked elevation of γ -GTP in comparison with the CDA-only group (2). The marked fibrosis was thought to be induced by severe hepatocyte necrosis and an indication of strong cytotoxicity by synergistic effects of DEN and CDA diet. Liver tissue

γ -GTP is known as a marker of hepatocellular enzyme-altered foci^{8-11,34}. Since a marked increase of GST-P-positive foci was observed in the CDAA+DEN-treated groups (4, 7, 8, and 9), increase in blood γ -GTP was thought to be a change related to the formation of hepatocellular enzyme-altered foci. Meanwhile, in the CRF-1+DEN-treated animals, the additional PB and PH treatment elevated the number of small foci, but slightly did the focal area. Since focal area in the groups treated with PH or PH+PB in addition to CRF-1+DEN (5, 6) showed much lower values than in the CDAA+DEN group (4), the promotion effect of PH and PB was thought to be much weaker than that of the CDAA diet.

Nowadays, 8-hydroxyguanine (8-OHG) has been a focus of attention as a new marker of carcinogenesis^{35,36}. 8-OHG has been reported that to be produced in vitro study when the C-8 position of guanine residues in DNA is hydroxylated by various oxygen radical producing agents³⁷. CDAA diet has been reported to induce linear increase in 8-OHG in liver cells from the first day of feeding²⁴. Therefore, CDAA diet is thought to induce reactive oxygen species (ROS)-derived oxidative stress to rat liver, presumably by inflammatory cells such as Kupffer cells or hepatocytes³⁸. PB also has been reported to produce reactive oxygen^{39,40}. Meanwhile, as for DEN used in this study, 8-OHG increases in liver cells several hours after administration of 0.001 mg/kg of DEN to F344 rats⁴¹. DEN is thought to be metabolized by P-450 in the liver, where it becomes a kind of cationic carcinogenic activator and damages DNA in the nucleus⁴². 8-OHG formation in the liver DNA by DEN is thought to be involved in initiation by DEN⁴¹. In recent years, the relation of hepatocyte necrosis and Ito cells to formation of cirrhosis has become clearer⁴³. When hepatocyte necrosis occurs, Ito cell proliferation factor is secreted, and inflammatory cells such as Kupffer cells gather and are activated^{44,45}. In the CDAA+DEN-treated groups (4, 7, 8, and 9), inflammatory cells were thought to be strongly activated by severe hepatocyte necrosis, and to produce reactive oxygen further and enhance CDAA diet's promotion effect.

These results showed that CDAA diet was more promotive than the PB and PH treatment in inducing hepatocellular foci formation initiated by DEN in rats, and the additional PB and PH treatment could not be promotive furthermore in the CDAA+DEN-treated animals.

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Fig. 2. Foci images from liver of F344 rat. GST-P stain. $\times 10$.

(a) CDAA (Group 2)	(e) CDAA+DEN (Group 4)
(b) CRF-1+DEN (Group 3)	(f) CDAA+DEN+PH (Group 7)
(c) CRF-1+DEN+PH (Group 5)	(g) CDAA+DEN+PB (Group 8)
(d) CRF-1+DEN+PH+PB (Group 6)	(h) CDAA+DEN+PH+PB (Group 9)

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