Long-term tocotrienol supplementation and glutathione-dependent enzymes during hepatocarcinogenesis in the rat

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The effects of long-term administration of tocotrienol on hepatocarcinogenesis in rats induced by diethyl nitrosamine (DEN) and 2-acetylaminofluorene (AAF) were investigated by the determination of plasma and liver gamma-glutamyl transpeptidase (GGT), cytosolic glutathione reductase (GSSG-Rx), glutathione peroxidase (GSH-Px) and glutathione S-transferase (GST). Twenty-eight male *Rattus norwegicus* rats (120–160g) were divided according to treatments into four groups: control group, tocotrienol – supplemented diet group (30mg/kg food), DEN/AAF-treated group and DEN/AAF-treated plus tocotrienol-supplemented-diet group (30mg/kg food). The rats were sacrificed after nine months.

The results obtained indicated no difference in the morphology and histology of the livers of control and tocotrienol-treated rats. Greyish-white neoplastic nodules (two per liver) were found in all the DEN/AAF treated rats (n=10) whereas only one nodule was found in one of the carcinogen treated rats receiving tocotrienol supplementation (n=6). Histological examination showed obvious cellular damage for both the DEN/AAF-treated rats and the tocotrienol-supplemented rats but were less severe in the latter.

Treatment with DEN/AAF caused increases in GGT, GSH-Px, GST and GSSG-Rx activities when compared to controls. These increases were also observed when tocotrienol was supplemented with DEN/AAF but the increases were less when compared to the rats receiving DEN/AAF only.

Introduction

The protective role of vitamin E against chemical carcinogenesis has been widely reported in various animal models[1]. However, although the protective ability of vitamin E has been studied extensively in colon and mammary tumours, very few studies have been carried out with liver tumours[2]. It is suggested that vitamin E protects against membrane damage resulting from uncontrolled peroxidation. Because many of the xenobiotic metabolizing enzymes are membrane-bound, conditions of lipid peroxidation may compromise the ability of animals to detoxify potentially harmful chemicals. Glutathione-dependent enzymes such as membrane-bound gamma-glutamyl transpeptidase (GGT), and cytosolic glutathione transferase (GST), glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-Rx), represent a group of phase II detoxification enzymes[3]. Glutathione transferases (EC 2.5.1.18) catalyze the conjugation reactions which represent a major pathway in cellular defense against toxic substances and in further metabolism of certain endogenous compounds. It has been suggested that this enzyme is useful as a marker of preneoplasia in chemical hepatocarcinogenesis[4]. The relationship between GST and chemical carcinogenesis has been widely reported[5].

GSH, GSH-Px and GST fulfil an important role in cellular defense mechanisms against hydrogen peroxide, organic peroxides and free radicals[6]. The reduction of peroxide is catalyzed by selenium-containing glutathione peroxidase (GSH-Px) (EC 1.11.1.9) and by other proteins which also exhibit GST activity. The metabolism of these reactive intermediates is associated with GSH oxidation and with the majority of the GSSG produced. Glutathione reductase (GSSG-Rx) (EC 1.6.4.2) maintains a high ratio of reduced glutathione (GSH):oxidized glutathione (GSSG) in the cell required in the protection of the cell from foreign compounds and the maintenance of important cellular functions[3].

GGT (EC 2.3.2.3) is a membrane-bound enzyme which is localized on the surface of the plasma membrane. The determination of GGT has been widely used as a marker of severity of hepatocarcinogenesis. GGT can either be determined histochemically or in liver homogenates[5,7].

In this study the effect of long-term supplementation

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of tocotrienol on hepatocarcinogenesis induced by diethylnitrosamine (DEN) and 2-acetylaminofluorene (AAF) was investigated in the rat. The protective effect of the tocotrienol was assessed by histology and by determination of plasma and liver microsomal gamma-glutamyl transpeptidase (GGT) and liver cytosolic glutathione transferases using two substrates, 1-chloro 2, 4 dinitrobenzene (CDNB) and 1, 2-dichloro 4-nitro benzene (DCNB), glutathione peroxidase, using two substrates (H_2O_2 and cumene hydroperoxide) and glutathione reductase.

Material and methods

Chemicals

A basal diet of rat chow was purchased from Gold Coin Co. Ltd (Malaysia). 2-Acetylaminofluorene, gamma-glutamyl carboxynitroanilide, glycylglycine, NADPH, H_2O_2, cumene hydroperoxide, reduced glutathione (GSH), oxidized glutathione (GSSG), 1, chloro-2, 4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB) and all other reagents used were of the highest commercial grade purchased from Sigma Chemical Co. (USA). The tocotrienol used was a gamma-enriched fraction (80% gamma-tocotrienol, 20% alpha and beta tocotrienols) of palm oil and supplied by Palm Oil Research Institute of Malaysia (PORIM).

Animal treatment

A total of 28 male *Rattus norwegicus* rats, each initially weighing 120–160 g and aged 7–8 weeks, were used. Rats were housed individually in wire cages in a ventilated room at 27 °C and maintained on normal or treated rat chow and water *ad libitum* for the duration of the experiments. Rats were divided into four groups: control (basal diet), tocotrienol-supplemented diet (30 mg/kg rat chow), DEN/AAF treated and a group treated with DEN/AAF and fed a diet supplemented with tocotrienol (30 mg/kg rat chow).

Hepatocarcinogenesis was induced according to the Solt and Farber method but without partial hepatectomy[8]. DEN was injected once intraperitoneally (ip) at a dose of 200 mg/kg body weight. After a recovery period of 2 weeks, the treated rats were fed with 0.02% (w/w) AAF for 2 months. Rats were started on the tocotrienol-supplemented diet 2 weeks after the DEN injection. A summary of the protocol is shown in Fig. 1. The rats were sacrificed after 9 months by cervical dislocation. The livers were immediately removed and weighed. Blood was collected in heparinized tubes, centrifuged at 2000 g in a bench centrifuge at 4 °C for 10 min and the plasma pipetted out and used immediately for GGT assays or stored at −70 °C.

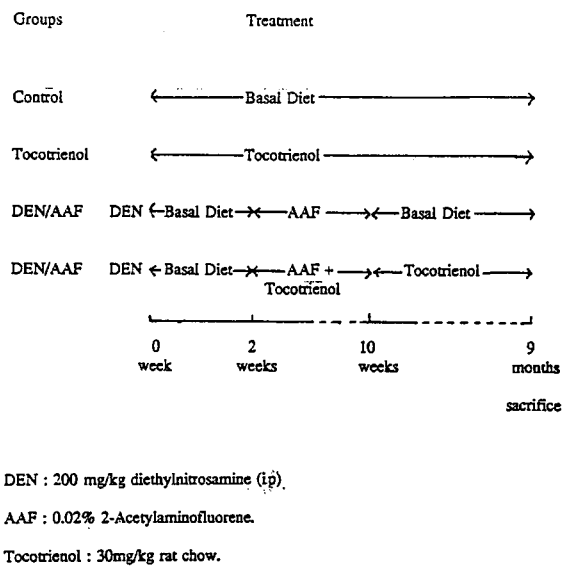


Fig. 1. Study protocol following Solt & Farber (1976)[8] with slight modification.

Histological examination was carried out by preparing slides of paraffin-embedded liver sections and staining with hematoxylin and eosin.

Cytosolic and microsomal fractions of the livers were prepared by the method of Speir and Wattenberg[9]. Briefly, rat livers were rinsed in 1.15% w/v potassium chloride (KCl). Tissues were cut into small pieces in 1.15% KCl at a volume of 3 ml of KCl per g liver and homogenized for 5 min in an Ultra Turrax homogenizer (Janker and Kunkel, FRG). The homogenate was centrifuged at 9000 g at 4 °C for 20 min in a Sorvall RC-5B superspeed centrifuge. The supernatant was pipetted into clean centrifuge tubes and centrifuged further at 104 000 g (35 000 rpm) at 4 °C in a Beckman L-60 centrifuge. The pellet obtained represents the microsomal fraction and was used for GGT assay. The cytosol was used for GST activities (using both CDNB and DCNB as substrates), GSH-Px activities (using H_2O_2 and cumene peroxide (CuOOH) as substrates) and GSSG-Rx activities.

Enzyme assays

Gamma-glutamyl transpeptidase was assayed by the method of Jacobs[10], with some modifications. Gamma-glutamyl carboxynitroanilide was used as substrate. The reaction mixture comprised 0.05 M Tris-HCl buffer pH 8.2 containing 2.9 mM substrate, 22 mM glycylglycine and 11 mM $MgCl_2$, in a total volume of 1 ml. Plasma (0.1 ml) was added and allowed to incubate for 45 min. The reaction was stopped by adding 5 ml 7.5 mM NaOH. The absorbance of the final mixture was measured at 405 nm. A blank using distilled water in place of the substrate was used as the serum blank and distilled water in place of serum was used as the substrate blank. Enzyme activities were expressed as iu/l where E_{405} $\mu M^{-1}cm^{-1}$ = 9.9 for p-nitroaniline. Microsomal GGT was also assayed in a similar way except that the microsomal pellet was resuspended in 5 volumes of 0.1M Tris-HCl buffer, pH 8.2, containing lmM $MgCl_2$. Microsomal GGT was expressed as iu/g protein.

Glutathione peroxidase GSH-Px activities were determined essentially by the method of Lawrence and Burk[11] with slight modifications using 0.8 ml of the reaction mixture pH 7.0, 0.2 mM NADPH, lmM NaN_3, lmM GSH and 1EU/ml GSSG-reductase. 0.1 ml of cytosol was added and the reaction started by the addition of 0.1 ml 15mM cumene hydroperoxide or 2.5mM H_2O_2 as substrate. Disappearance of NADPH at

340nm was recorded at 29 °C. Specific activity was defined as the oxidation of 1 μmole NADPH $min^{-1}mg^{-1}$ protein. The extinction coefficient of NADPH used was 6.22 $mM^{-1}cm^{-1}$.

Glutathione reductase GSSG-Rx activities were measured by the method of Racker[12] with slight modifications. The assay mixture consisted of 0.1M phosphate buffer pH 7.6, 0.1mM NADPH, 0.5 mM EDTA, 1 mM GSSG and cytosol in a final volume of 1.0 ml. The enzyme activity was quantitated at 29 °C by measuring the disappearance of NADPH at 340 nm. Specific activity for GSH-Rx was defined as the oxidation of 1 μmole of NADPH $min^{-1}mg^{-1}$ protein.

Glutathione S-transferase The activities of GST in the liver cytosol were assayed according to the method of Habig et al.[13] using 1-chloro-2, 4-dinitrobenzene (CDNB) or 2,4 dichloro-1-nitrobenzene (DCNB) as the second substrate. The reaction mixture consisted of 0.1 M phosphate buffer pH 6.5 (pH 7.5), 1 mM GSH, 1 mM CDNB (or DCNB) and cytosol in a final volume of 1.0 ml. The reaction was followed in a Shimadzu 2101 PC spectrophotometer at 340 nm. One unit of GST activity is expressed as the amount of enzyme required to conjugate one μmole of the second substrate with GSH per minute at 29 °C. Specific activity was defined as units of enzyme per mg protein in the cytosol. Protein was assayed by the method of Bradford[14].

Statistical analysis
The results obtained were analysed using one way analysis of variance and Student's t-test. A value of $P<0.05$ was considered significant.

Results

No difference was observed in the morphology of the livers of control and tocotrienol-treated rats. Two nodules per liver were found in all the DEN/AAF-treated rats (10/10) whereas only one nodule was found in one of the DEN/AAF-plus-tocotrienol-treated rats (1/6) (Table 1). Histological examination showed no difference in the livers of control and tocotrienol-treated rats (Figs 2a and 2b). However, obvious cellular damage was evident for both the DEN/AAF treated rats (Figs 3a and 3b) and the rats receiving DEN/AAF with tocotrienol supplementation (Figs 4a and 4b) but was less extensive in the latter. The hepatocytes of DEN/AAF-treated rats were paler, more vacuolated and had lost their normal shape and arrangement. The portal triads were less obvious.

Tocotrienol had no effect on the plasma and liver microsomal GGT (Table 1). DEN/AAF increased plasma and liver GGT activity compared to that of controls ($P<0.01$–$P<0.001$). However when tocotrienol was supplemented in the diet of DEN/AAF-treated rats, GGT activity was significantly less than in rats treated with DEN/AAF only.

Rats treated with the carcinogens DEN/AAF also showed increased cytosolic GST activities using the two substrated, CDNB and DCNB (Table 2). Rats supplemented with tocotrienol and treated with carcinogens showed increases in this enzyme, but the increases were less than those receiving carcinogens only ($P<0.05$–$P<0.001$).

Table 1. Effect of DEN/AAF and tocotrienol on plasma and liver gamma-glutamyl transpeptidase (GGT) after 9 months. Values are means ± S.E.M.

	Plasma GGT (iu/l)	Liver GGT (iu/g protein)	No. of rates with nodules
Control (n=6)	5.00±0.65	5.68±0.30	None
Tocotrienol (n=6)	5.50±0.57	5.41±0.46	None
DEN/AAF (n=10)	19.80±2.70**	27.71±2.14**	10/10
DEN/AAF +Tocotrienol (n=6)	9.69=0.75*+	16.86±1.41*+	1/6

Significantly different from control group *$P<0.01$, **$P<0.001$ and DEN/AAF group: +$P<0.01$.

Table 2. Effect of DEN/AAF and tocotrienol on liver cytosolic glutathione S-transferase after nine months using substrates CDNB and DCNB. Values are means ± S.E.M.

	CDNB (μmol/min/mg protein)	DCNB (μmol/min/mg protein)
Control (n=6)	0.65±0.05	0.011±0.001
Tocotrienol (n=6)	0.65±0.02	0.012±0.001
DEN/AAF (n=6)	1.23±0.09**	0.026±0.001*
DEN/AAF+Tocotrienol (n=6)	0.97±0.06*+	0.017±0.001++

Significantly different from control group *$P<0.01$, **$P<0.001$ and DEN/AAF: +$P<0.05$, ++$P<0.001$.

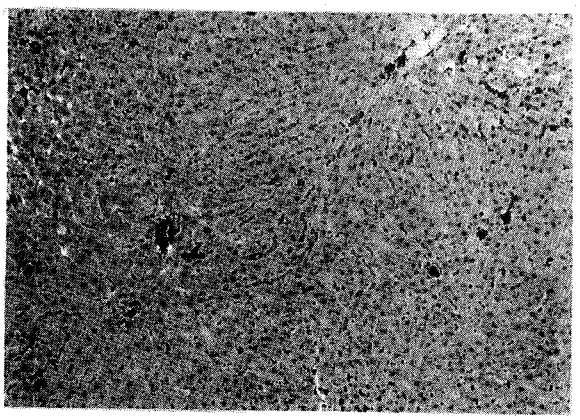
Treatment with tocotrienol did not cause any change in the GSH-Px activities using the two substrates H_2O_2 and cumene hydroperoxide (Table 3) when compared to controls. DEN/AAF-increased GSH-Px activity using H_2O_2 as the substrate but only a slight non-significant increase in liver GSH-Px activity using cumene hydroperoxide as substrate. Supplementation with tocotrienol also increased the GSH-Px (H_2O_2) to values in between those in control and DEN/AAF-only-treated rats ($P<0.05$). However with cumene hydroperoxide as the substrate, the increase in GSH-Px activity was not

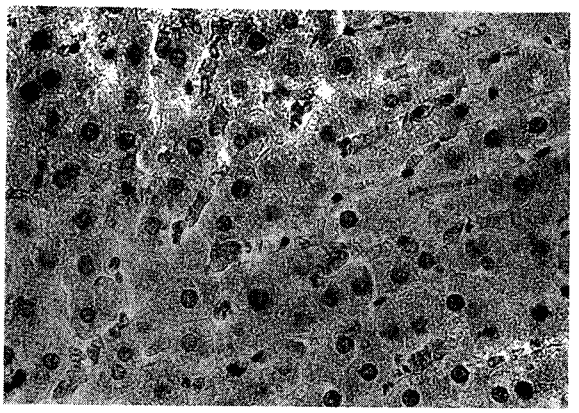
Table 3. Effect of DEN/AAF and tocotrienol on liver cytosolic GSH-Px and GSSG-Rx at the time of sacrifice. Values (μmol/min/mg protein) are means ± S.E.M.

	GSH-Px		GSSG-Rx
	H_2O_2	Cu OOH	
Control (n=6)	0.59±0.02	1.10±0.03	0.17±0.01
Tocotrienol (n=6)	0.64±0.13	1.10±0.02	0.18±0.01
DEN/AAF (n=10)	0.71±0.02***	1.13±0.03	0.21±0.01**
DEN/AAF+ Tocotrienol (n=6)	0.62±0.04+	1.10±0.04	0.19+0.01*

Significantly different from control group *$P<0.05$, **$P<0.02$ and ***$P<0.01$ DEN/AAF + $P<0.02$.

(a)

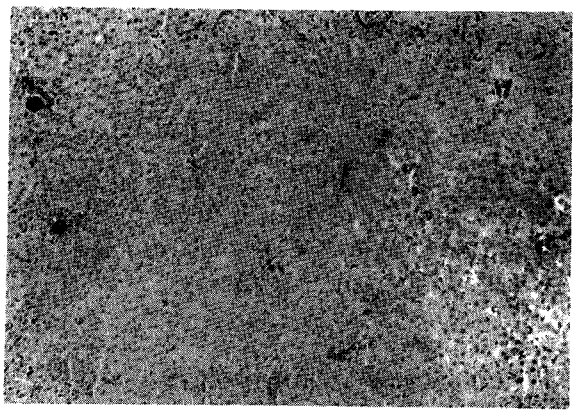


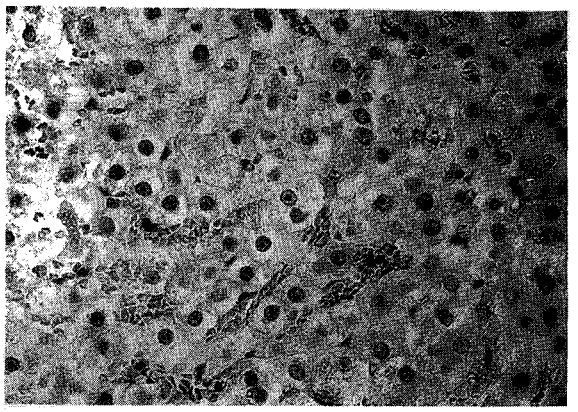


(b)

Figure 2 (a & b). Photomicrograph (50x and 200x) of control liver. Normal organization and arrangements of hepatocytes. The portal triads were obvious. The cell membranes were obvious and the nuclei round and smooth.

(a)

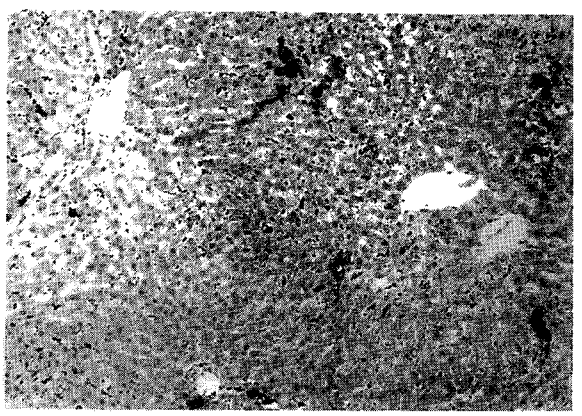


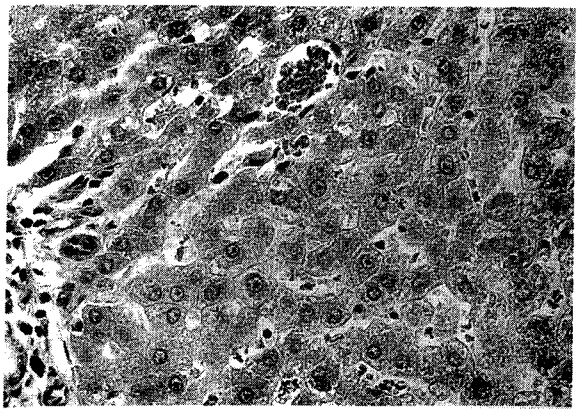


(b)

Figure 3 (a & b). Photomicrograph (50x and 200x) of DEN/AAF treated rat liver. The hepatocytes were paler than control liver. There were disorganization of architecture and the portal triads were less obvious. The cell membranes were not obvious. The section (3b) exhibiting an altered hepatic focus was noted by the large pale cytoplasm and large nuclei in the group of cells in the centre.

(a)





(b)

Figure 4 (a & b). Photomicrograph (50x and 200x) of rat liver treated with DEN/AAF and supplemented with tocotrienol. Organization of hepatocytes were towards normal cells. The portal triads were obvious. The damage was less extensive with clearly visible cells membranes.

significant when compared to control rats or DEN/AAF treated rats.

DEN/AAF also increased GSSG-Rx activity when compared to control and tocotrienol-treated rats (Table 3). When tocotrienol was supplemented in the diet with DEN/AAF, GSSG-Rx activities also increased above control values but were lower than in rats receiving DEN/AAF only ($P<0.02$).

Discussion

Most of the studies concerning vitamin E and chemical carcinogenesis have involved the use of tocopherol as the source of vitamin E[1]. This was probably due to the available supply of tocopherol. However, tocotrienols are now more readily available and its commercial production from palm oil is a possibility in the near future. It would be of interest to see whether tocotrienols have the same protective effect against hepatocarcinogenesis. When evaluated for activity against several transplantable murine tumours tocotrienols were effective against sarcoma 180, Ehrlich's carcinoma, IMC carcinoma and Meth A fibrosarcoma, but they had no antitumour activity against P-388 leukemia. Alpha-tocopherol had only a slight effect against sarcoma 180 and IMC carcinoma[15]. In this study, the gamma-tocotrienol-enriched fraction of palm oil used as a supplement in the diet was shown to have a protective effect on hepatocarcinogenesis. Gould et al.[16] showed a significant increase in tumour latency when mammary tumours were induced by 7, 12-diethylbenza(a) anthracene and supplemented with tocotrienol.

Increase in GGT activity with DEN/AAF treatment was also observed in the liver. Supplementation with tocotrienol resulted in less of an increase in GGT activity in both the plasma and liver in the rats treated with tocotrienol in addition to DEN/AAF. In humans, plasma GGT activity has been reported useful as a marker of neoplasia and to correlate well with the extent of cancer diseases[17]. In animal studies, the degree of severity of cancer process is directly proportional to the enzyme activities[7,18].

Changes in molecular forms of hepatic cytosolic GST during rat chemical hepatocarcinogenesis were investigated by Kitahara et al.[19]. GST activities toward substrates CDNB and DCNB increased with the increased area of GGT-positive foci and hyper plastic nodules induced by DEN followed by AAF plus hepatectomy. GST types A and P were markedly increased and induced in livers bearing foci and nodules. These enzymes are the preneoplastic enzymes for chemical hepatocarcinogenesis[19,20]. This study also showed increases in GST activities for both substrates whereas tocotrienol supplementation caused a decrease in the activities, suggesting a protective effect of tocotrienol.

Kitahara et al.[19] suggested that GST-B acts like GSH-Px in hyperplastic nodules partly to compensate for the decreased Se-dependent GSH-Px. From our results GSH-Px activities increased, but were not significant when cumene hydroperoxide was used as the substrate. When tocotrienol was supplemented in the diet of rats treated with DEN/AAF, the activities decreased to values in between the control and rats treated with DEN/AAF only. A similar pattern was observed with GSSG-Rx activity.

In conclusion, the glutathione-dependent enzymes GST, GSH-Px and GSSG-Rx were increased with DEN/AAF treatment. Supplementation with tocotrienol brought these enzymes to values in between control and DEN/AAF treatment, suggesting a protective role of tocotrienol. This observation was further supported by a similar pattern of increases in GGT activities and by histological examination.

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Long-term tocotrienol supplementation and glutathione-dependent enzymes during hepatocarcinogenesis in the rat

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長期補充生育三烯酚對大白鼠肝致癌作用的良性影響及其谷胱甘肽依賴酶的變化

摘要

作者用二乙基亞硝胺（DEN）和2－乙酰氨基芴（AAF）引致大白鼠肝癌，同時觀察長期補充生育三烯酚的作用，并研究了對血漿和肝髒的γ－谷氨酰轉肽酶（GGT），胞漿的谷胱甘肽還原酶（GSSG-RX），谷胱甘肽過氧化物酶（GSH-PX）和谷胱甘肽S－轉移酶（GST）的影響。

作者用28只Rattus Norwegicus雄鼠(120-160克)爲對象，分成四組：對照組，生育三烯酚補充組(30毫克/公斤飼料)，DEN/AAF組和DEN/AAF加生育三烯酚組(30毫克/公斤飼料)。九個月后把大白鼠處死，結果顯示對照組與生育三烯酚補充組大鼠的肝髒没有形態學和組織學的差异。在 DEN/AAF 組大鼠 (n=10) 肝髒均有兩個灰白腫瘤結節，而 DEN/AAF 并補充生育三烯酚的大鼠 (n=6) 肝髒，則只有一個灰白腫瘤結節，組織學檢驗顯示 DEN/AAF 組和 DEN/AAF并補充生育三烯酚組均有明顯的細胞損害，但後者程度較輕。

DEN/AAF組的GGT，GSH-PX，GST 和 GSSG- RX 活性均高於對照組。這種升高在DEF/AAF 并補充生育三烯酚組亦可見到，但程度較 DEN/AAF 組輕。