Jpn. J. Vet. Res. **47**(1–2) : 13–23 (1999)

FULL PAPER

Effects of concurrent exposure to 3–methylcholanthrene and vitamin A on fetal development in rats

El. Bohi M. Khlood[1,2], Hiroyuki Miyoshi[3], Hisato Iwata[1], Akio Kazusaka[1], Yasuhiro Kon[3], ALi H. Abou Hadid[2], El. Kelish Moustafe[2], Mervat H. Ghonim[2], and Shoichi Fujita[1,*]

(Accepted for publication : July. 26, 1999)

Abstract

To investigate the effect of the environmental pollutants, polycyclic aromatic hydrocarbons (PAHs), on retinoic acid-induced teratogenesis, all-*trans*-retinoic acid (RA) dissolved in corn oil (120 mg/kg) was administered orally to pregnant rats at the 11th day of gestation with and without the prior intraperitoneal treatment with 10 mg/kg 3-methylcholanthrene (3-MC) for 3 days. Dams were killed on the 20th day of pregnancy. The examinations of fetuses revealed that 3-MC barely enough to cause induction of P-450 in pregnant dams had profound embryo-toxic effects : the fetal resorption amounted to ~60% of total number of implantations. The fetuses survived weighed less than the control fetuses. All of RA-treated mothers had fetuses with abnormalities, and the main malformations were absence of tail (100%), caudal and sacral malformations (100%), and cleft palate (42%). Pregnant dams received both 3-MC and RA had a reduced severeness of tail anomaly (33%), while the rest, 67%, had short vestigial tail. Caudal and sacral malformations were detected but at a milder degree. We did not observe cleft palate in this group. The concurrent treatment of dams with 3-MC and RA led to an increased inducibility of cytochrome P-450 and subsequently, CYP1A1 dependent enzyme activity higher than those observed after the injection of 3-MC alone. UDP-glucuronyl-transferase activity was also markedly induced in concurrent 3-MC and RA group higher than that in 3-MC alone. We suggest that the induction of P-450 and alteration of metabolic enzyme activities may play an important role in reducing the teratogenic potency of RA. However, RA-treatment did not retard the embryo-toxic effect of 3-MC but rather potentiated.

Keywords : All-trans-retinoic acid (RA), Cytochrome P-450., Drug metabolism., Fetal malformation., 3-Methylcholanthrene (3-MC)

[1] Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan.

[2] Department of Pharmacology and Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Zagazig University, Egypt.

[3] Laboratory of Experimental Animal Science, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan.

[*] Coreresponding author, Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan. Tel. 81–11–706–6948. Fax. 81–11–706–5105.

Introduction

The first human teratogen, identified epidemiologically only as recently as the 1940s, was the rubella virus. This was followed in the early 1960s by the widely published limb reduction defects due to thalidomide use in Europe. An equally shocking but somewhat less well-known example of environmental teratogenicity involves severe central nervous system (CNS) abnormalities due to methyl mercury pollution of the fish supply of the Minamata Bay, Japan in the 1950s and 1960s[1].

Retinoids, metabolites of retinoic acid (RA), have been of interest to developmental biologists for many decades because of their teratogenic effects on fetal development. Many studies have found that retinoic acid given during pregnancy leads to many birth defects[2,3] while other studies indicate that a diet deficient in RA is likewise teratogenic[4]. Thus, normal development seems to require a careful balance of retinoid concentrations. Retinoids are a family of low molecular weight, hydrophobic molecules derived from RA. These compounds exhibit striking effect on the growth and differentiation of many types of cells. Retinoids are also used clinically in the treatment of some types of cancer[5] and in some dermatological diseases[6]. All-*trans*-retinoic acid (tretinoin) appears to be the active form of RA in all tissues of vertebrate except in the retina. Much of the work on teratogenic effects of retinoids has focused on the role of all-*trans* retinoic acid, a biologically active RA derivative.

The normal requirement of RA for adults is supplied by an adequate diet. The rational uses for retinol are for the treatment of RA deficiency and as prophylaxis in high-risk subjects during periods of increased requirement, such as infancy, pregnancy and lactation. During pregnancy and lactation, it is advisable to increase the maternal intake of RA by about 25%[6]. Otherwise once RA deficiency has been diagnosed, intensive therapy should be instituted.

Estrogens and oral contraceptives elevate the plasma concentrations of retinoid binding protein (RBP), but the effect of pregnancy is complex. During the first trimester, the mean content of retinol in plasma falls, followed by a slow rise and a return to normal at parturition. It is likely that the increased demands for retinol lead to its withdrawal from the blood at a rate exceeding that of its mobilization from the liver. The placental barrier prevents the extensive transfer of retinol or carotinoids. Studies in animals have suggested that transplacental transport of RBP occurs during early pregnancy, therefore the fetus begins to prepare its own RBP[6].

Kistler and Howard[7] studied the relationship between the day of retinoid treatment and the development of malformation. They reported that embryo lethality and malformations of the head occur between day 8 and 10 of gestation (head malformation includes exencephaly, encephalocele, open eyes, cleft palate and facial and cranial abnormalities).

Fujii et al.[4] identified a novel cytochrome P-450 (P-450-RA) that specifically metabolizes RA, in vitro. P-450-RA converts all-*trans* retinoic acid to 5, 8-epoxy all-*trans* retinoic acid, it also can metabolize other biologically active RAs such as 9-cis-RA and 13-cis-RA but it fails to metabolize its precursors such as retinal and retinol. They also remarked that in post-implantation embryos between 9.5 and 10.5 dpc, P-450-RA is expressed mainly in caudal neural plate, hind gut and tail bud (region 1) and in neural crest cells for neural ganglia (region 2). The same authors provided several lines of evidences indicating that P-450-RA can convert biologically active forms of RA to inactive forms, so one can say that the levels of active RA in P-450-RA-expressing cells would be lower than those in non-expressing cells. Using P19 and

Hela cell lines, it was demonstrated that P-450 can metabolize all-*trans* retinoic acid present in culture medium. Endogenous all-*trans* RA and 9-cis RA are present at about 10-2 μM in embryo and adult organs, therefore these P-450 expressing cells can metabolize physiological levels of RA. Jay et al.[8] reported a new P-450 from zebra fish (referred to as P-450RAI) that can metabolize RA. The amino acid sequence of P-450RAI showed significant homology to P-450-RA of Fujii et al.[4] (about 60% of the amino acid residues are identical). However these two P-450s have different activities : P-450RAI metabolizes all-*trans* RA to 4-oxo and 4-hydroxy all-*trans*-RA while P-450RA metabolizes all-*trans*-RA to 5,8 epoxy all-*trans* RA. Jey et al.[9] also described the cloning and characterization of the first mammalian RA inducible cytochrome P-450 (P-450-RAI), which belongs to a novel class of cytochromes (CYP26). This novel cytochrome is responsible for the metabolic activities as in zebra fish. They also demonstrated that P-450-RAI is inducible by RA in a number of different cell types, so it plays an important role in determining the metabolic fate of endogenous retinoids and may also be implicated in the clearance of exogenous retinoids administered therapeutically.

In this study we try to detect the effect of prior exposure of the pregnant mother to an inducer of drug metabolizing enzymes on the teratogenic potency of retinoic acid.

Materials and methods

Animals

Six-week-old, three-day-pregnant female Wistar rats of about 160 g body weight were purchased from Japan SLC, Inc (Shizuoka, Japan). They were kept in stainless steel cages in a full air conditioned room. Pellet diet (Labo MR Stock, Nihon Nosan Industries Ltd, Kanagawa, Japan) and tap water were available ad libitum.

Chemicals

All-trans-retinoic acid (RA) and 3-methylcholanthrene (3-MC) were obtained from Wako-Pure Chemical Industries Ltd, (Tokyo, Japan). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADPH were purchased from Oriental Yeast Co. (Tokyo, Japan). UDP-glucuronic acid (UDPGA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents and solvents used were commercially available and of the highest quality.

Chemical administration and collection of samples

The pregnant rats were grouped in 4-groups ; rats of the first group were treated once with 120 mg/kg body weight all-*trans* retinoic acid (RA) dissolved in corn oil by the oral intubation at the 11th day of pregnancy. The time schedule of RA administration and its dose were chosen according to Kistler[10] to obtain optimal teratogenic effect with lower resorption percentage. Pregnant female rats in the second group were treated with 3-MC dissolved in corn oil at the seventh day of pregnancy by intraperitoneal injection of a daily dose of 10 mg/kg/day for three days. The pregnant rats of the third group received both RA and 3-MC at the same time schedule as previous groups. Rats of the fourth group (control group) received only corn oil either orally or intraperitoneally. Either chemical-treated or control pregnant female rats were killed by decapitation in the 20th day of gestation and their fetuses were removed by caesarean section, and the number of sites of implantations and resorption were recorded. The fetuses were weighed, sexed, and examined for external malformations, eviscerated, and stained with alizarin red and alcian blue according to Inouye[11], and finally stored in glycerine. Using a dissecting microscope with a camera, malformation pattern in fetuses was recorded.

Preparation of microsomes

The dam's livers were excised, perfused

with ice-cooled 1.15% KCl (w/v). Liver microsomes were prepared according to Omura and Sato[12]. Microsomal protein concentrations were determined according to Lowry et al.[13], using bovine serum albumin as the standard. Total microsomal cytochrome P-450 was quantified from the CO difference spectrum of the dithionite-reduced proteins between 490 and 450 nm using an extinction coefficient of 91 $mM^{-1}cm^{-1}$.

Enzyme assay

Drug metabolizing enzyme activities in microsomes were measured. Reactions were carried out under the optimal conditions regarding incubation temperature and protein concentrations. Ethoxyresorfin O-deethylase activity, CYP 1A1 dependent activity in rats was assayed according to Clark et al.[14]. UDP-glucuronyl transferase activity was determined according to Bock et al.[15] using p-nitrophenol as the substrate.

Western blotting analysis

Liver microsomal proteins from pregnant female rats were separated by electrophoresis in a 10% sodium dodecyl sulfate-polyacrylamide gel Laemmli[16]. Immunoblotting analysis of microsomal proteins against CYP 1A1 antiserum was carried out according to Towbin et al.[17]. Spectral configurations of western blots were analyzed using the method described as NIH image according to Lennard[18].

Statistical analysis

Statistical comparisons were made by analysis of variance, followed by Fisher's protected least significant difference test. The value p less than 0.05 was regarded as statistically significant.

RESULTS

Reproductive performance

Resorption

Single oral dose of RA administered to pregnant wistar rats on gestation day 11 led to a little resorption rate reaching 20% of either early or late resorption (Fig. 1), while this rate increased significantly to about 60% and 84% in 3-MC and 3-MC plus RA groups, respectively (Table 1).

Percentage of alive fetuses

Mean percentage of mothers having alive fetuses at the term was significantly decreased to about 61% after intraperitoneal injection of 3-MC and only 30% in 3-MC and RA group while oral administration of 120 mg/kg RA in the 11th gestation day revealed 100% of dams had fetuses at term.

Mean fetal weight

Mean fetal weight significantly dropped in

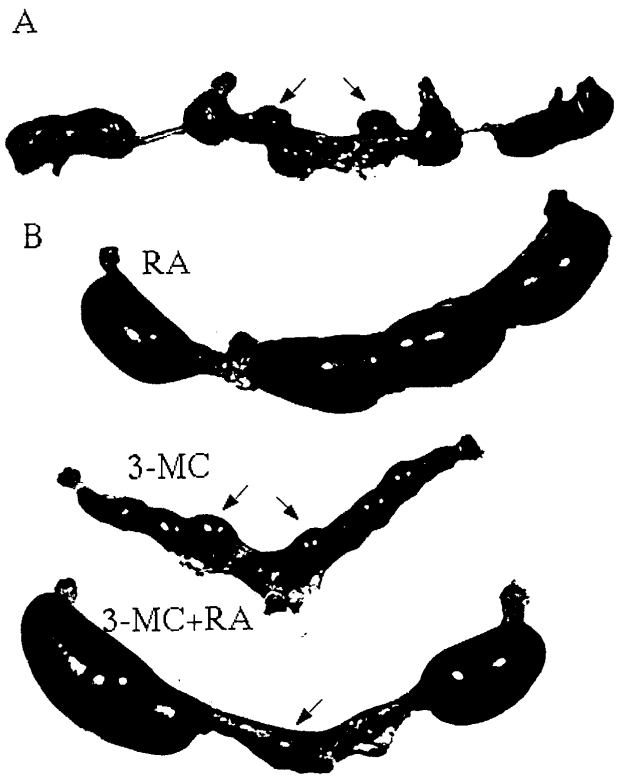

Figure 1.

A. Uterus of pregnant dams (3-MC + RA) killed at 20 th day of pregnancy showing early and late resorption (arrow).

B. Uterus of pregnant dams killed at 20th day of pregnancy. The photo shows the uterus from dams treated with RA and / or 3-MC.

RA : retinoic acid, 3-MC : 3-methylcholanthrene, arrows indicate resorption sites.

Table 1. The resorption rates of fetuses at 3–MC and /or RA treatment.

Group	$\frac{\text{T. R.}^{1}}{\text{T. I.}^{2}}$ %	$\frac{\text{D. R.}^{3}}{\text{T. D.}^{4}}$ %	$\frac{\text{D. A. F.}^{5}}{\text{T. D.}^{4}}$ %	$\frac{\text{T. A. F.}^{5}}{\text{T. I.}^{2}}$ %	Fetal weight g±SD
Control	6	26	84	74	2.8±0.6
3–MC	60	80	61	36	2.2±0.3
RA	20	40	100	80	2.03±0.2
3–MC+RA	84	100	30	14	2.9±0.7

[1] T. R. : Total resorption. [2] T. I. : Total implantation. [3] D. R. : Dams with resorption. [4] T. D. : Total number of dums. [5] D. A. F. : Dams with alive fetuses. [8] T. A. F. : Total number of alive fetuses.

Table 2. Malformations caused by 3–MC and /or RA treatment.

Group	Tail anomaly %		Caudal and sacral malformation %	Cleft palate %
	Anury	Vestigial		
Control	0	0	0	0
3–MC	0	0	0	0
RA	100	0	100	42
3–MC+RA	33	67	74	0

the groups administered RA or 3-MC alone, while that in the group received concurrent treatment of RA and 3-MC was similar to that in the control.

Malformation pattern

Malformations of fetuses from mothers of various treatment groups were summarized in Table 2. The complete absence of tail (anury) was observed in 100% of fetuses of dams received RA (Figs. 2 and 3). Malformations in caudal and sacral vertebrae (Fig. 4) were found in all fetuses, cleft palate was in 42% of fetuses, and malformations such as wavy ribs, shortened long bones, absence of ossification center in lumber vertebrae and failure of ossification were in few cases of the same group (Fig. 5).

The incidence of anury was decreased in fetuses from dams received both RA and 3-MC as compared with that in the group of dams received RA alone. The number of fetuses without tail reduced to 33% as compared with 100% in RA treatment alone, while the remaining 67% of fetuses were with malformed short tail (vestigial-tail) (Figs. 2 and 3). Although the incidence of the caudal and sacral vertebral malformations was high (74%), the fetuses of this group showed milder extent of malformations (Fig. 4) than those observed in RA treated dams. Cleft palate was not observed in this group, and in general, fetuses of this group suffered from poorly ossified bones indicated by their staining behavior (not stained by alizarin red but stained by alcian blue). In total, in spite of the high incidence of fetal resorption, 3-MC treatment appeared to have

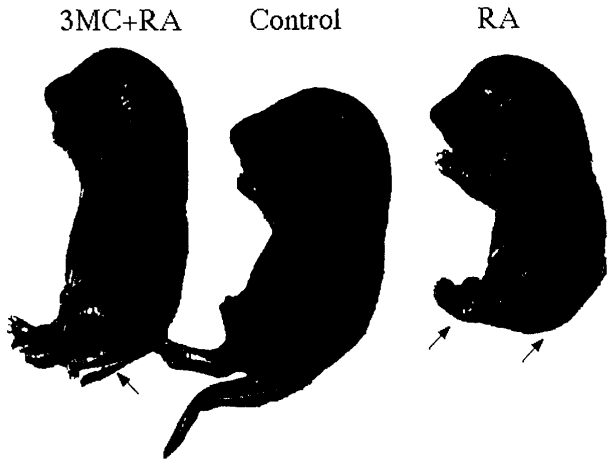

Figure 2.
Full term fetuses obtained at 20th day of pregnancy. Arrows indicate absence of tail, short hind limb in fetuses from RA administered dams and short vestigial tail in fetuses from 3-MC+RA-injected dams.

Figure 3.
A. Skeleton of fetuses from control dams.
B. Tail anomaly (anury) induced by RA on 11th day of gestation in rats.
C. Malformed short tail in fetuses from dams treated with 3-MC and RA. Fetuses were obtained by caesarean section on 20th day of gestation and processed for skeletal staining with alcian blue and alizarin red. ×70.

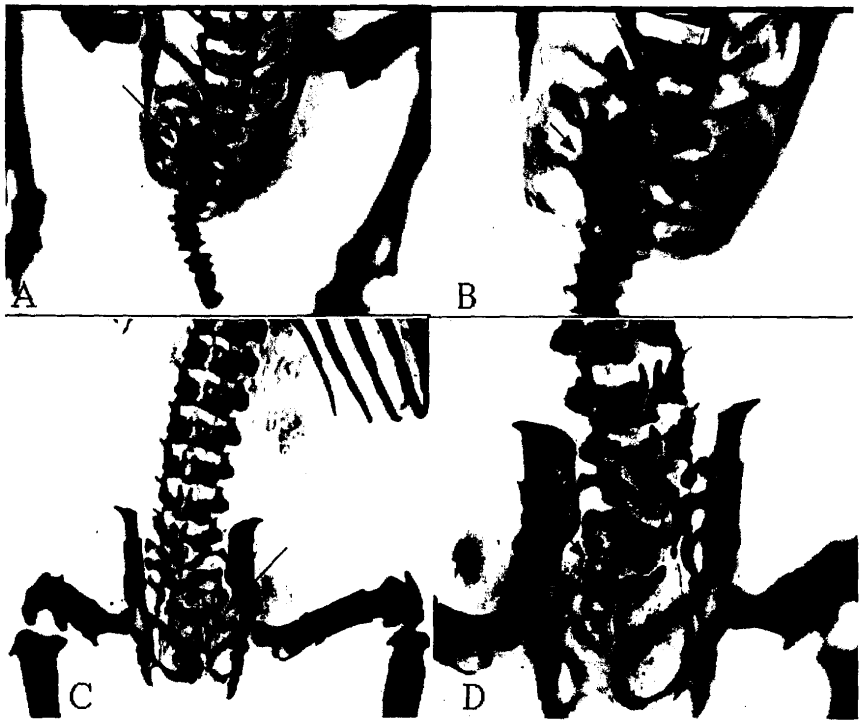

Figure 4.
A and B. Malformation of caudal and sacral vertebrae in fetuses from dams administrated with 3-MC and RA. A : 70×magnification power, B : × 100.
C and D. Malformation of caudal and sacral vertebrae in fetuses from RA administered dam. C : ×70, D : ×100.

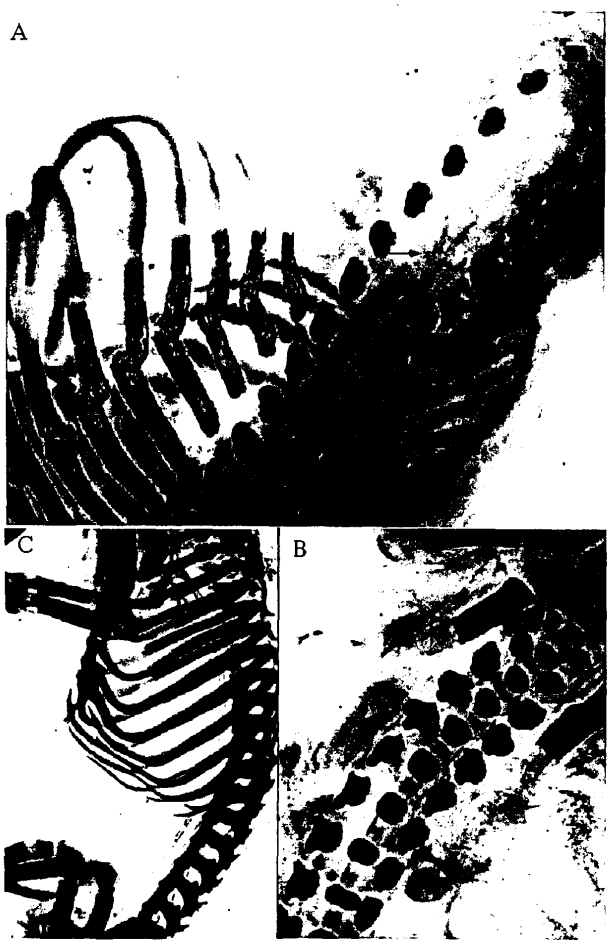

Figure 5.

A. Wavy ribs in fetuses of pregnant dams after intubation once with 120 mg/kg 3-MC (arrow). The same photo shows absence of ossification centers in lumber vertebrae (arrow). ×70.

B. Normal ribs in fetuses from control dams at term. × 70.

C. Normal lumber vertebrae in fetuses from control dams at term. ×70.

reduced the teratogenic effect of RA in alive fetuses from 3-MC+RA-treated dams. No malformations could be observed in 3-MC injected dams beside fetal resorption.

Biochemical results

Induction of P-450

Cytochrome P-450 was significantly induced in either groups received 3-MC, but the rats received 3MC+RA showed higher induction levels than the rats in 3-MC group, while in RA group P-450 levels dropped sinificantly (Fig. 6A). The liver from pregnant 3-MC-treated rats showed 10 fold higher activity of ethoxyresorfin O-deethylase (EROD) than control (Fig. 6B).

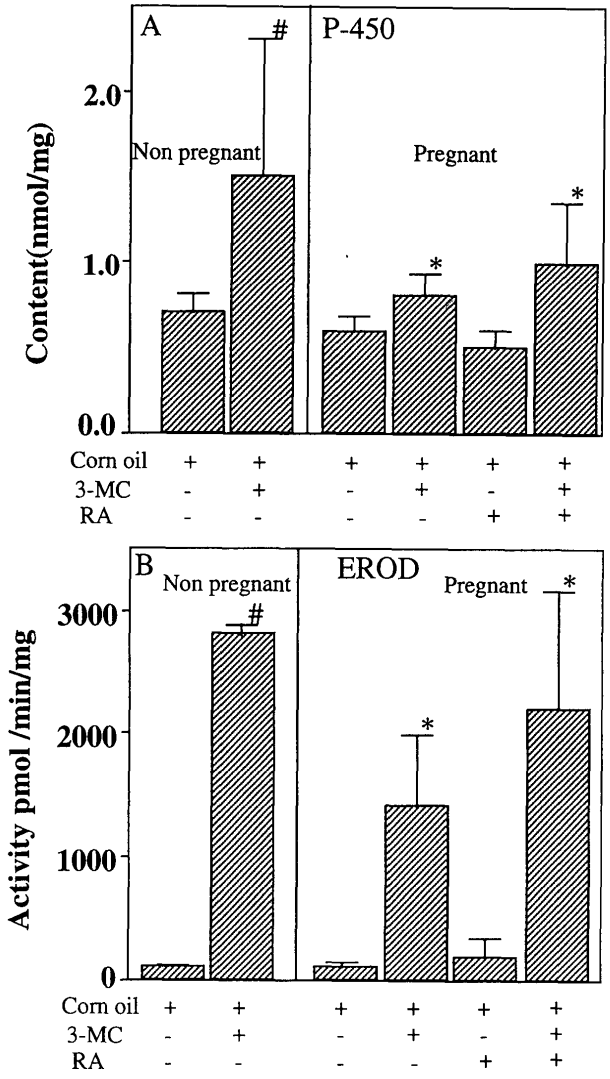

Figure 6.

A. Cytochrome P450 levels in hepatic microsomes from non-pregnant and pregnant dams at term. The cytochrome P450 content was calculated from the CO-difference spectra of reduced microsomes. Each value represents the means±SD.

B. Ethoxyresorfin O-deethylase activity in hepatic microsomes of non-pregnant and pregnant Wistar rats at term. Each value represents the means±SD.

3MC : 3-methylcholanthrene, RA : all-*trans*-retinoic acid, P450 : cytochrome P-450, EROD : Ethoxyresorfin-O-deethylase.

significantly different from control non-pregnant.

* significantly different from all other pregnants.

The treatment with RA did not alter EROD activity. The group received both RA and 3-MC showed 18 fold induction of EROD activity in comparison to control pregnant rats. The activity of UDP-glucuronyl transferase (UDPGT) was significantly lower in control pregnant rats than in non-pregnant female rats (Fig. 7). In RA treated dams the enzyme activity was more or less similar to control dams. UDPGT levels were elevated in rats received 3-MC alone (1.7 fold) and markedly elevated in dams treated both with 3-MC and RA (2.2 fold) in comparison with non-treated pregnant rats. The induction level was not significantly different from 3-MC-injected non-pregnant Wistar rats.

To verify that the enzyme activities correlated with the levels of immunodetectable CYP1A1, western blotting was performed using CYP1A1 antibody (Fig. 8). CYP1A1 expression level markedly elevated in the rats received 3-MC and RA (14 fold), while its level was 6 fold in dams received 3-MC alone. RA group did not show any sign of alteration. In non-pregnants, CYP1A1 was markedly induced after 3-MC injection (7 fold).

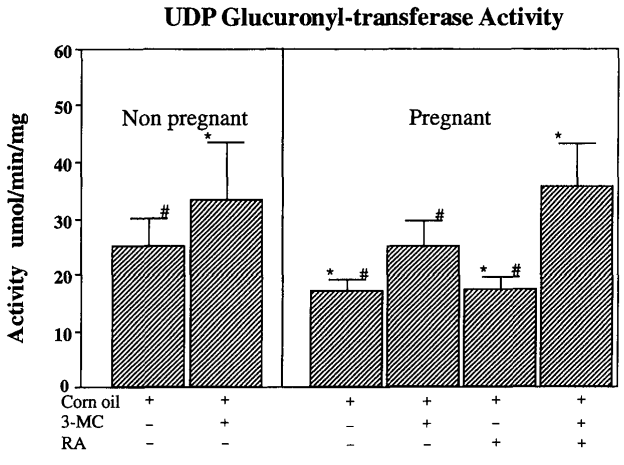

Figure 7.
UDP-glucuronyl transferase activity in hepatic microsomes from female Wistar rats. Each value represents the means ± SD.
* significantly different from control non-pregnant.
# significantly different from 3-MC non-pregnant females.

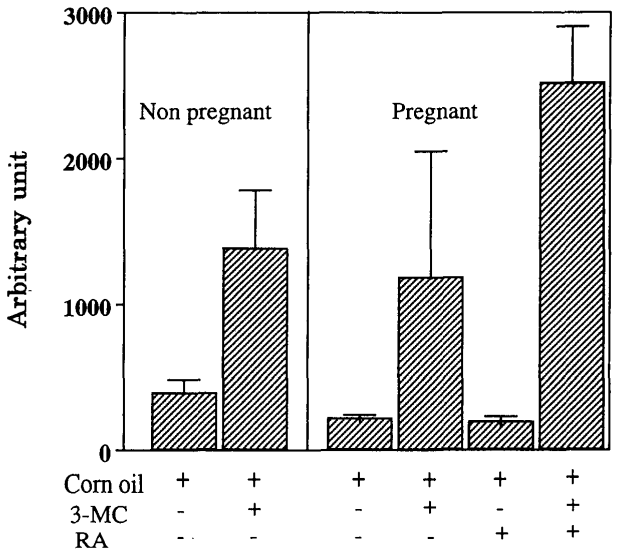

Figure 8.
Western blotting analysis of hepatic microsomes from female Wistar rats. The microsomal proteins were electrophoretically separated on 10% SDS PAGE gels and transfered to the nitrocellulose membrane. Blots were reacted with CYP1A1 antibody and visualized by the enzyme-linked peroxidase system. Concentration of CYP1A1 was estimated from the NIH image analysis. Each value is plotted in arbitrary unit and represents the means ± SD.

Discussion

The polycyclic aromatic hydrocarbons (PAHs) are a class of compounds widely distributed in the environment. Most PAHs in the environment result from combustion processes, either from mobile sources such as internal-combustion engines associated with transportation vehicles or from stationary sources such as fossil fuel or wood combustion for heat and power generation[19].

About 150 compounds which could be characterized as PAHs were found in tobacco smoke. Chemical constituents of tobacco readily cross the placenta and produce various forms of embryo toxicity such as small-for-date babies and intrauterine demise (miscarriage and stillbirth). Furthermore, the combined effect of intrauterine growth retardation and premature delivery, perhaps coupled with 10-fold increase in

the rate of infant mortality in the smoking mothers[20].

3-MC, one of PAH family was used in this study to measure its hazardous effect on pregnant dams. We applied 3-MC with the dose 10 mg/kg/day for 3 days, which did not produce any signs of toxicity in the mothers in the second trimester of pregnancy (Organogenesis stage) to pregnant Wistar rats. Severe embryo-toxicity was the main common observation in fetuses from 3-MC-treated mothers. Embryo-toxicity produced were either resorption (early or late resorption) or reduced fetal body weights. The mechanism of 3-MC fetal toxicity was not clear. The dose of 3-MC used to treat pregnant mothers was enough to induce P-4501A1 and related drug metabolizing enzyme activities in the liver of the mother rats. In human, reduced fetal weight and the induction of P-4501A1 were noted in the placenta of the smoking mothers. A number of PAHs including 3-MC is known to be metabolically activated by P-4501A1 to more reactive metabolic intermediates. These intermediates are often mutagenic and carcinogenic, that is to say that they are genotoxic. In non-pregnant rats, these intermediates may be effectively eliminated by the pleiotropically induced phase II drug metabolizing enzymes such as UDPGT. However, our results indicate that UDPGT activity towards p-nitrophenol was markedly reduced in pregnant rats. The ability to induce UDPGT activity upon 3-MC treatment was not retarded in pregnant rats.

The ability of excess RA to induce malformations has been known for at least 40 years[21]. The effect on the developing embryo is widespread including malformations of the face, limbs, heart, central nervous system and skeleton. In our study, single oral administration of 120 mg/kg all-*trans*-RA to pregnant dams on the 11th day of pregnancy led to potent teratogenic effects in fetuses; 100% of fetuses with anury tail and caudal and sacral malformations, while cleft palate was observed in 42% of fetuses.

In this study we aimed to measure the effect of concurrent exposure of pregnant women to PAHs from our polluted environment or from smoking and excessive vitamin A therapy. We observed that dual treatment of pregnant rats with 3-MC and RA led to the increased fetal resorption (60% at 3-MC alone, and 84% at 3-MC plus RA) and the decreased teratogenicity of retinoids; only 33% of fetuses had anury and we could not observe cleft palate which was observed in the group treated with RA alone. The mean fetal body weight was similar to that of control rats.

Cytochrome P-450 may have an important role in reducing the teratogenicity of retinoids. Previous studies of Fujii et al.[4] and Jay et al.[8] identified a novel P-450 (P-450-RA and P-450I) which converts all-*trans*-RA to 5, 8 epoxy all-*trans* retinoic acid; P-450RA can convert biologically active forms of RA to inactive forms leading to minimizing the RA-teratogenicity. We think that the reduced occurrence of the fetal malformation by RA after concurrent treatment with 3-MC may be due to the induction of P-450 by 3-MC. P-450 1A1 and 1A2 are typically induced by 3-MC. They may have the ability to transform RA to less teratogenic form[4,8]. Alternatively, 3-MC may induce P-450RA[4,8].

In contrast, the induction of cytochrome P450 1A1 may have detrimental effect on the developing fetuses. The inductive effect of P-450 1A1 by 3-MC was more pronounced in the group received concurrent RA administration, and so was the resorption of the embryo induced by 3-MC treatment. Our results indicate that UDPGT activity towards p-nitrophenol was markedly reduced in pregnant RA treated rats; this condition supposed to be more severe in human due to less extensive glucuronidation of RA and limited formation of 4-oxo-RA metabolite[22]. 3-MC led to significant elevation of UDPGT which can partially improve tera-

togenicity with no effect in embryo-toxicity.

Further study is needed to elucidate the mechanism of improvement of RA teratogenicity by 3-MC treatment and the increase in 3-MC induced embryo-toxicity by RA treatment.

Acknowledgements

This work was supported in part by a Grant-in Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan to S.F.(No. 04404019) and from the Ministry of High Education , Egypt.

References

1) Kipen M. H. and Zuber C. 1994. Occupational and environmental impacts on reproductive health. Ann. New York Acad. Sci., 736: 58–73.
2) Lynberg, M. C., Khoury, M. J., Lammer, E. J., Waller, K. O., Cordero, F. F. and Erickson J.D. 1990. Sensitivity, specificity and positive predictive value of multiple malformations in isotretinoin embryopathy surveillance. Teratology, 42: 513–519.
3) Rutledge, J. C., Shourbaji, A. G., Hughes, L. A., Polifka, J. E., Cruz, Y. P., Bishop, J. B. and Generoso, W. M. 1994. Limb and lower body duplications induced by retinoic acid in mice. Proc. Natl. Acad. Sci. USA. , 91: 5436–5440.
4) Fujii, H., Sato, T., Kaneko, S., Gotoh, O., Kuriyama, Y. F., Osawa, K., Kato, S. and Hamada, H. 1997. Metabolic activation of retinoic acid by a novel P-450 different expressed in developing mouse embryos. EMBO, 16: 4163–4173.
5) Lippman, S. M., Benner, S. E. and Hong, W. K. 1993 Chemoprevention.Strategies for the control of cancer. Cancer, 72: 984–990.
6) Marcus, R. and Coulston, A. 1996. Fat soluble vitamins. In: *Goodman and Gilman's, Pharmacological Basis of Therapeutics*, 9th ed., McGraw-Hill, 1574–1582.
7) Kistler, A. and Howard, B. 1990. Testing of retinoids for teratogenicity in vivo. Methods in Enzymology, 190, 433–435.
8) Jay, A. W., Guo, Y. D., Baetz, K., Jones, B. B., Bonasoro, J., Hsu, K. E., Dilworth, F. J., Jones, G. and Petkovich, M. 1996. Identification of the retinoic acid-inducible all-*trans*-retinoic acid 4-hydroxylase. J. Biol. Chem., 271: 29922–29927.
9) Jey, A. W., Jones, B. B., Guo, Y. D., Dilworth, F. J., Bonasoro, J., Jones, G. and Petkovich, M. 1997. DNA cloning retinoic acid-metabolizing enzyme (P-450RAI) identifies a novel family of cytochromes P-450 (CYP26). J. Biol. Chem., 272: 18538–18541.
10) Kistler, A. 1981. Teratogenesis of retinoic acid in rats: Susceptible stages and suppression of retinoic acid-induced limb malformations by cycloheximide. Teratology, 23: 25–31.
11) Inouye, M. 1976. Differential staining of cartilage and bone in fetal mouse skeleton by alcian blue and alizarin red. S. Cong. Anom., 16: 171–173.
12) Omura, T. and Sato, R. 1964. The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem., 239: 2370–2378.
13) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the foline phenol reagent. J. Biol. Chem., 193: 265–275.
14) Clark, M. A., Bing, B. A., Gottschall, P. E. and Williams, J. F. 1995. Differential effect of cytokines on the phenobarbital or 3-Methylcholanthrene induction of P-450 mediated monooxygenase activity in cultured rat hepatocytes. Biochem. Pharmacol., 49: 104–106.
15) Bock, K. W., Burchell, B., Dutton, G. J., Hanninen, O., Mulder, J., Owens, I. S., Siest, G. and Tephly, T. R. 1983. UDP-glucuronyl transferase: Guidelines for consistent interm terminology and assay conditions. Biochem. Pharmacol., 32: 953–955.
16) Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680–685.
17) Towbin, H., Staehlin, T. and Gordon, J. 1979. Electrophilic transfer of of proteins from

polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA., 76: 4350–4354.

18) Lennard, P. 1990. Image analysis for all. Nature , 347: 103–104.

19) Grimmer, G. 1983. *Environmental carcinogens*: polycyclic aromatic hydrocarbons, CRC Press, Boca Raton, FL.

20) Seegmiller, E R. 1997. Selected examples of developmental toxicants. In: *Comprehensive Toxicology* 10, pp.29–36. Sipes, G., Mcqueen, C.A. and Gandolfi, J. eds., Elsevier Science Ltd.

21) Cohlan, S Q., 1954, Congenital anomalies in the rat produced by excessive intake of vitamin A during pregnancy. Pediatrics, 13: 556–567.

22) Clarke, O. D. 1997. Pharmacokinetic and structure-activity considerations. In: *Comprehensive Toxicology* 10, pp.505. Sipes, G., Mcqueen, C.A. and Gandolfi, J. eds., Elsevier Science Ltd.