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Effects of ovulen-50, diethylnitrosamine and phenobarbital on liver regeneration in female rats

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Abstract. Short term effects of ovulen-50, a combination type oral contraceptive agent and phenobarbital—an established hepatic tumour promoter, were examined in the livers of diethylnitrosamine-initiated and uninitiated female rats. Liver mitotic activity as judged by liver weight, [^{3}H] thymidine incorporation into DNA and levels of DNA, RNA and protein were measured in non-regenerating and regenerating liver. Hepatic γ-glutamyl transpeptidase activity and hepatocyte agglutination with concanavalin A were examined in diethylnitrosamine- and/or phenobarbital-treated rats.

The results indicate that diethylnitrosamine or ovulen-50 individually are mitoinhibitory in regenerating liver. Phenobarbital alone has a slight mitostimulatory effects in non-regenerating liver, but no effect on liver regeneration. Administration of ovulen-50 and phenobarbital to diethylnitrosamine initiated rats mitigated the mitoinhibition during regeneration. Contrary to the earlier observation with ovulen-50, neither phenobarbital nor diethylnitrosamine induced hepatocyte agglutination in the presence of concanavalin A. Like ovulen-50, diethylnitrosamine also increased the level of hepatic γ-glutamyl transpeptidase. Phenobarbital produced only insignificant rise and did not substantially exacerbate the effect diethylnitrosamine.

The data show that though some of the effects of ovulen-50 resemble those of diethylnitrosamine or phenobarbital, the changes observed may not be related to the neoplastic phenomenon since they were not seen in an initiator-promoter combination regimen.

Keywords. Oral contraceptive agent; phenobarbital; diethylnitrosamine; liver regeneration; nucleic acids; γ-glutamyl transpeptidase; concanavalin A; hepatocytes agglutination.

Introduction

An association between the use of estrogens and higher incidence of hepatocellular adenoma and possibly carcinoma has been reported. A number of studies have been conducted to clarify the role of estrogens in neoplasia. In some studies estrogens were found to act as complete carcinogens when fed to animals as estrogen-progestin combination for up to 104 weeks. The results varied with the species, strain and estrogen dose (Schardein *et al.,* 1970). Other studies show that estrogens are not themselves carcinogens, but promote the effects of carcinogens (Yager and Yager, 1980).

There are very few studies wherein the effects of steroidal contraceptives on the liver have been examined from a functional point of view using biochemical parameters. Also most of the earlier studies have employed very high doses of hormones.

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Abbreviations used: OCA, Oral contraceptive agent; pH, partial hepatectomy; GGT, *y*-glutamyl transpeptidase; Con A, concanavalin A; DEN, diethylnitrosamine; PB, phenobarbital.

An increased accumulation of fat after carbontetrachloride ($CC1_4$) injury in the livers of Enovid-treated compared to untreated control female rats has been reported (Joshi and Rao, 1969). However, the recovery of the liver following CCl_4 injury though delayed by Enovid was found to be complete. In another study on female rhesus monkeys, it was observed that the increase in serum enzymes of hepatic origin seen after the administration of aflatoxin a powerful hepatocarcinogen could be prevented by the simultaneous administration of ovulen an oral contraceptive agent (OCA) (Belavady *et al.,* 1973). These results were supported by the work of Mg bodile and Holscher (1976), who found that the pre-administration of 100 μg ovral-28 for 16 days mitigated the acute effects of a single dose of aflatoxin B_1 in rat liver.

Studies of Mukundan *et al.*· (1981) show that daily administration of 5 μg ethinylestradiol, 100 μg ethynodiol diacetate in the form of OCA-ovulen-50 to female rats leads to an increase in liver lipids and mitostimulation in a non-regenerating liver, but mitoinhibition in liver regenerating after partial hepatectomy (pH). We have recently shown that after similar treatment, there is a slight increase in the level of the tumour marker enzyme γ-glutamyl transpeptidase (GGT) and increased agglutinability of isolated hepatocytes with concanavalin A (Con A) suggesting certain degree of liver damage and/or transformation in OCA-treated female rats (Annapurna *et al*·*,* 1987).

Inhibition of cell proliferation and/or DNA synthesis is also brought about by many carcinogens and this is considered to have some significance in the carcinogenic process (Farber, 1976).

The studies reported in this paper were designed to find out if established tumour initiator and promoter compounds like diethylnitrosamine (DEN) and phenobarbital (PB) respectively would behave like OCA with regard to the above cited effects, when administered singly and in combination over a short duration of 7 weeks.

Experimental

Ovulen-50 was obtained from Searle Company, Bombay. DEN from Sigma Chemicals, St. Louis, Missouri, USA, and phenobarbital from Indian Drugs and Pharmaceuticals, Hyderabad. [^{3}H] Thymidine was obtained from Bhabha Atomic Research Centre, Bombay. All other compounds used were of the highest purity available.

Two experiments were carried out. In the first experiment 64 weanling female rats of Wistar/NIN strain were divided into two equal groups. One group received a single intraperitoneal injection of DEN (200 mg/kg body weight) in saline (initiated rats). The other group received the same quantity of saline and served as uninitiated control. Four weeks after this injection, 16 rats from DEN-initiated group and 16 from saline-treated group received either propylene glycol or one-tenth tablet of the OCA-ovulen-50 as propylene glycol suspension for 7 weeks. Three or four rats from each of the four treatment groups—(i) control, (ii) DEN, (iii) OCA, (iv) DEN + OCA-treated were sacrificed at 0, 24, 48 and 72 h after pH. pH was performed by removing two third of the liver under light ether anesthesia (Higgins and Anderson, 1931). One hour before sacrificing, 5 μCi of [^{3}H]thymidine (sp. act. 9·8 μCi/mmol) was injected through the tail vein. After sacrificing the rats, the livers were rapidly removed and examined for total protein, DNA, RNA and [^{3}H]thymidine incorporation into DNA by the method described earlier (Mukundan *et al.,* 1981).

A portion of the non-regenerating liver was used for histopathological studies by conventional methods and 6 μm paraffin sections were stained and examined.

Throughout the experiment, the animals were fed a balanced diet containing 20% casein, 63·8% starch, 10% groundnut oil, salt and vitamin mixture from the time of weanling till sacrifice. They were housed in individual screen-bottom cages and maintained at 22-25°C in 12 h light-dark cycle. Body weights were recorded at weekly intervals.

The design of the second experiment and parameters measured were similar to the first experiment except that in place of OCA, 0·05% PB was administered through diet. The animals were sacrificed at 0 and 24 h after pH. The 24 h time point was used since maximum effect on liver regeneration with OCA treatment was seen at that time point. In addition to the mitotic activity, levels of GGT and hepatocyte agglutination with Con A were also examined in non-regenerating livers by methods described earlier (Annapurna *et al.,* 1987).

Statistical analysis of the data was done by using analysis of variance with or without log transformation of the values and Duncan's multiple range test, considering data on the effects of treatment and time after partial hepatectomy at various time points together.

Results

[^3H] Thymidine incorporation and levels of DNA, RNA and protein in non-regenerating and regenerating liver.

In the first experiment the mitostimulatory effects of OCA on non-regenerating liver and the mitoinhibitory effect on regenerating liver reported earlier from our laboratory were confirmed (figure 1) (Mukundan *et al.,* 1981; Annapurna *et al.,* 1987). DEN alone had similar effects of greater magnitude. Surprisingly, however, when OCA was given to DEN-initiated rats there was no exacerbation. On the contrary, mitoinhibition after pH seen with each of these agents individually, was completely mitigated when they both were administered and the process of regeneration was found to be normal in these rats (figure 1).

Results of the second experiment show that PB *per se* tended to increase the [^{3}H]-thymidine incorporation into non-regenerating liver, suggesting stimulation of mitotic activity. However, this trend was not significant in the liver weight and DNA, RNA and protein contents (figure 2). PB had no effect on regeneration of liver after partial hepatectomy. When administered to DEN-initiated rats, PB mitigated the effect of DEN on non-regenerating as well as regenerating liver (figure 2).

Effects on liver GGT activity

DEN produced significant rise in liver GGT activity. PB showed a similar but non-significant trend. The GGT activity was highest in rats which had been initiated with DEN and subsequently treated with PB, but this increase was not significantly more than DEN-treatment alone (table 1).

Hepatocyte agglutination with Con A

DEN or PB singly or in combination showed no effect on agglutination of hepatocytes with Con A.

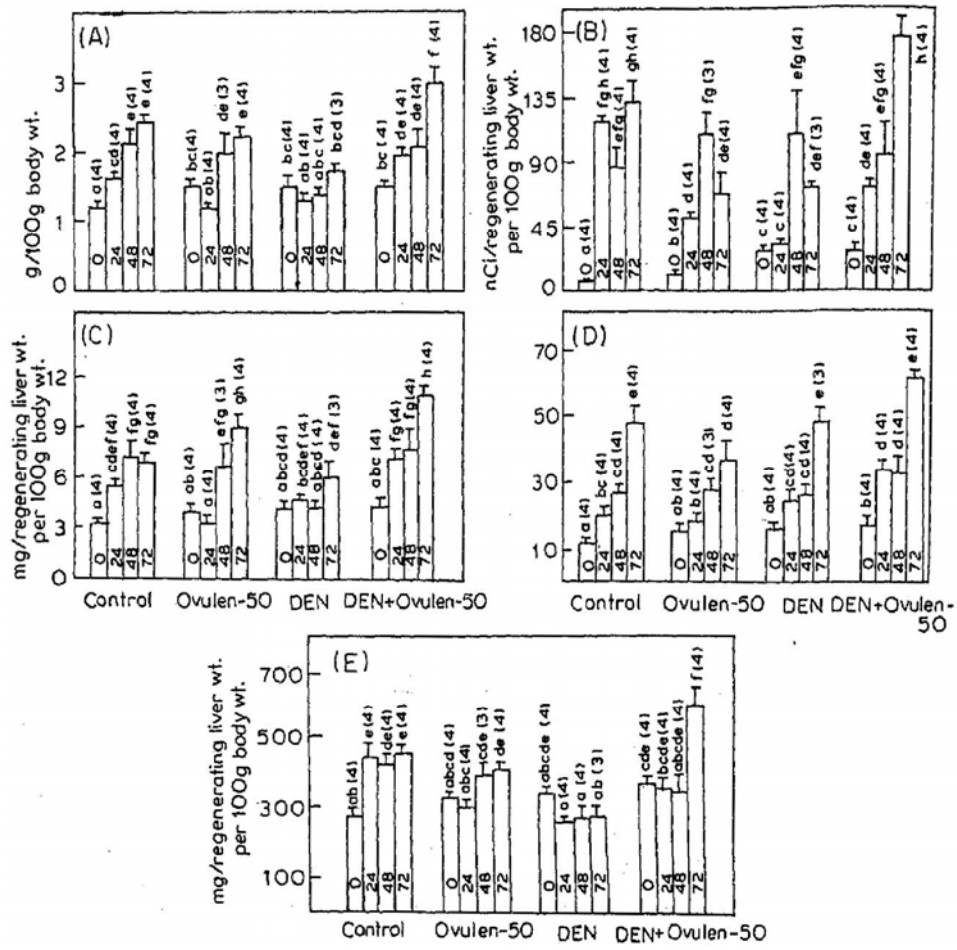

Figure 1. Liver regeneration in DEN and/or ovulen-50 treated female rats. (A), Liver weight; (B), [^{3}H]thymidine incorporation into liver DNA; (C), DNA; (D), RNA; (E), protein.

Results are presented as mean ±SE for groups of 3–4 rats. Statistical analysis of the data was done by using analysis of variance with or without log transformation of the values and Duncans multiple range test, using the data on the effects of all the treatments and time points after partial hepatectomy together. Bars not sharing even one common superscript a, b, c, d, e are significantly different (P<0·05). 0, 24, 48 and 72 represent time in hours after pH. Numbers in parentheses indicate number of animals used.

Histology

The livers of rats from the OCA or PB-treated groups were not remarkably different from the controls. However, livers of animals given DEN alone or in combination with PB or OCA exhibited a few scattered foci of vacuolated cells.

Discussion

Earlier studies from our laboratory had shown that treatment of female rats with OCA, ovulen-50 produced certain changes in the liver which are reminiscent of

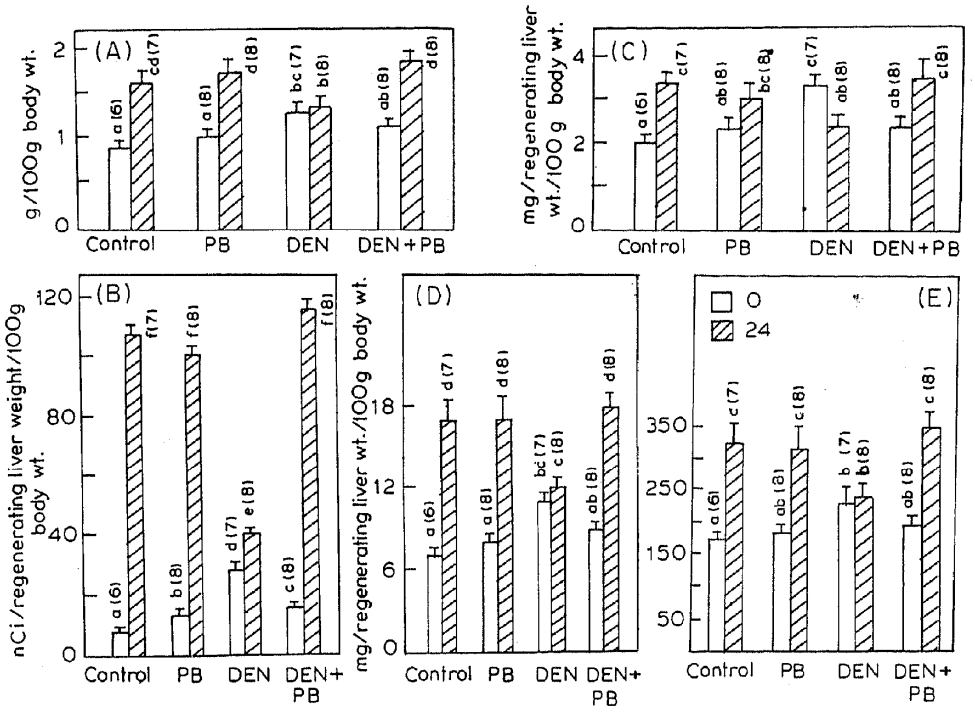

Figure 2. Liver regeneration in DEN and/or PB treated female rats. (A), Liverweight; (B), [^{3}H]thymidine incorporation into liver DNA; (C), DNA; (D), RNA; (E), protein·

Results are presented as ±SE for groups of 7-8 rats. Statistical analysis of the data was done by using analysis of variance with or without log transformation of the values and Duncans multiple range test, using the data on the effects of all the treatments and time points after pH together. Bars not bearing even one common superscript are significantly different (P<0·05). 0 and 24 represent time in hours after partial hepatectomy. Numbers in parentheses indicate number of animals used.

Table 1. Effect of PB treatment for 7 weeks on GGT activity in the liver of DEN initiated and uninitiated female rats.

	Treatment			
	Control	PB	DEN	DEN + PB
GGT activity				
Total activity nmol/mt/g liver	143·10 ± 9·902^a (6)	161·62 ± 10·279^{ab} (8)	187·59 ± 14·261^{bc} (7)	222·22 ± 19·279^c (8)
Specific activity nmol/mt/mg protein	0·763 ± 0·043^a (6)	0·883 ± 0·074^{ab} (8)	1·093 ± 0·116^{bc} (7)	1·233 ± 0·109^c (8)

Values are mean ±SE·
Numbers in parentheses indicate the number of animals used·
Data analysed by analysis of variance and Duncan's multiple range test.
Values bearing even one common superscript are not statistically significant·

hepatocarcinogens. Thus OCA treatment produced mitostimulation in non-regenerating liver but mito-inhibition in regenerating liver (Mukundan *et al.*, 1981).

It raised the levels of the tumour marker enzyme GGT in the liver and made hepatocytes susceptible to agglutination by Con A (Annapurna *et al.,* 1987).

The object of the present investigation was to find out if these biochemical and morphological changes can be labelled as early markers of neoplastic phenomenon and thereby ascertain the neoplastic risk of OCA in a short duration study. A two stage initiator-promoter model of hepatic tumourogenesis as described by Wanless and Medline (1982) was used in which either OCA or PB (a known tumour-promoting agent) were administered to female rats which had been initiated with DEN (a known tumour-initiating agent). Suitable control and single-treatment groups were also included for comparison.

The data reported show that the action of OCA on several of the parameters such as mito-stimulation of non-regenerating liver, mito-inhibition of regenerating liver and increase in liver GGT activity (Annapurna *et al.,* 1987) resembles that of DEN, a tumour initiator. Yet the first two effects were not seen in rats treated with DEN-OCA combination or an established initiator promoter combination like DEN and PB.

Hepatocyte agglutination with Con A earlier reported for OCA-treated rats was also not observed with DEN and/or PB treated rats. From these observations it appears that the observed changes may not be related to the neoplastic phenomenon and cannot be used as early markers of tumourogenicity.

Impaired liver regeneration after partial hepatectomy has been observed in conditions such as vitamin A deficiency (Jayaram *et al.,* 1975), some alkaloid treatment (Downing and Peterson, 1968) and alcohol treatment (Lieber, 1981) in rats. These treatments are known to produce liver damage but not hepatic neoplasia.

Membrane changes brought about by OCA as reflected in hepatocyte agglutination with Con A may not have any bearing to the neoplastic process in view of the fact that similar hepatocyte agglutination with Con A treatment is also brought about under some non-carcinogenic conditions like the adaptive changes to diet and drugs (Henriquez *et al.,* 1979; Nicolson *et al.,* 1986). Thus the agglutination noted with OCA-treated rat hepatocytes could be a result of an adaptive change.

The 2-fold rise in GGT activity of OCA treated hepatocytes observed earlier (Annapurna *et al.,* 1987) is of a lesser magnitude than the 15-150-fold increase seen in tumour cells. An increase in hepatic GGT activity of a smaller magnitude has been reported in other conditions of liver damage such as alcoholism and following use of drugs such as phenytoin, aminopyrine, etc. (Goldberg 1980). The slight increase in serum alkaline phosphatase (Annapurna *et al.,* 1987) together with an increase in GGT activity in liver may indicate a small degree of hepatic damage, perhaps mild cholestasis.

It is therefore possible that under the conditions of experimentation, OCA-ovulen-50 is hepatotoxic and not neoplastic in female rats.

The observation that alterations produced by each of these agents DEN, OCA and PB when administered singly were not exaggerated and even abolished when given as initiator-promoter combination regimen is difficult to explain. It may be related to an interplay of drug metabolizing enzymes induced by these agents (Lapis *et al.,* 1984; Sweeny and Cole, 1980; Schulte-Hermann, 1979; Shenfield, 1986) and consequent drug disposal. The conclusion that OCA may be hepatotoxic and not

neoplastic is substantiated by the results of a study recently reported by us wherein OCA when administered for a longer period of 60 weeks in an initiator promoter regimen, failed to produce neoplasia (Annapurna *et al.,* 1988).

The rat model to study the effects of OCA used in the present study has been used in several earlier studies involving different types of OCA effects (Ahmed and Bamji, 1976; Mukundan *et al.,* 1981; Annapurna *et al.,* 1987; Vijayalakshmi and Bamji, 1987). Though on the body weight basis the dose of hormones employed is almost 25 times the human dose this cannot be regarded as a very high dose since the effective contraceptive dose of hormones on the basis of body weight is 10 times higher for rats as compared to humans (Banik and Revesz, 1968). Metabolic disposal of these hormones is much faster in rats than in humans (Schwenk *et al.,* 1979).

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