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HORMONAL INFLUENCE ON OOCYTIC DEPLETION INDUCED BY 7,12-DIMETHYL BENZANTHRACENE (DMBA)

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PREFACE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi-110 067. The work is original and has not been submitted so far, in part or full, for any other degree or diploma of any University.

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CONTENTS

HORMONAL INFLUENCE ON OOCYTIC DEPLETION INDUCED BY 7, 12 - DIMETHYL-BENZANTHRACENE (DMBA)

INTRODUCTION

The objective studies on cancer is to achieve a better understanding of the factors responsible for their initiation, development, and ultimately their eradication and control. Since the turn of the century it has been accepted that hormones play an essential role in different types of cancers such as breast cancer, ovarian cancer, hepatoma, bladder carcinoma etc. There are many evidences to prove that hormones play an important role in the inhibition or promotion of cancers. Estradiol 17β inhibits spon - taneous hepatoma in mice (Agnew and Gardner, 1952), transplanted walker carcinoma of rat 256 (Stock and Sugiura, 1958) and trans - planted mammary carcinoma of mice (Hircherberg, 1963). Testosterone is found to inhibit transplanted adrenal tumour of mice (Browning et. al., 1959), myosarcoma of uterus R3234 of guinea pig (Dunning, 1960). And the hormone cortisone inhibits solid sarcoma (Stock and Sugivora, 1958, Hirschburg, 1963), malignant lymphoma and ascites hepatoma (Oakasu and Tateus, 1963) of mice. For the activation of tumours by hormones the following examples can be cited. Prolactin and insulin are found to promote breast cancer (Mainwaring, W.I.P.

and Mangan, P.R., 1973; Cohen, N.D. and Hilf, R., 1974). Estrogen was found to promote the renal tumours in hamsters. (Algand, F.T., 1960).

Inhibition and promotion of tumour growth by various hormones is already reviewed extensively by many. (Ralph I. Dorfman, 1965; W.V. Gardner, 1976; Paul Franchimont, 1975; Russel Hilf et. al., 1976); Robinson et. al., 1968; Muller, 1971; Jensen et. al., 1972; O'Malley and Means, 1972; Pitot and Yatuin, 1973; Cuatrecasas, 1974; King and Mainwaring, 1974). But the physiological mechanism of interaction of hormones with hormonal cancers is yet to be explained.

And now-a-days, having seen the interaction of hormones with cancer it seems definitely possible to have an endocrine therapy for it. And the concept of feedback regulation of the several hypothalmic, hypophyseal and organ axes is essential in finding out a rational endocrine therapy for cancer (Kelly H. Clifton, 1977). The present investigation is related to the hormonal interactions with the early events taking place in the ovary, prior to the tumour development.

Compared with other tissues there are a number of advantages in using the ovary as a model of studying cancer. The organ is a well defined entity consisting of a variety of different cell types and

normally well defined morphological and functional inter - relationships exhibiting a progression in differentiation. Much is already known of the function of different ovarian cells and there are demonstrable relationships between the ovary and its response to other endogeneous factors. And the functional capacity of the ovary is influenced by a wide variety of hormones such as follicular stimulating hormone (FSH), lutenising hormone (LH), insulin, estrogen, progeterone, and prolactin. So ovary seems to be the best model to study the interaction of hormones with tumours.

This topic is very Words detailed

As noted earlier, the ovary is consisting of a variety of different cell types. The outer covering of the ovary is formed of an epithelial lining, the germinalepithelium underneath which lies the tunica albuginea, a connective tissue layer. Follicular apparatuses, stromal and connective tissues, intertitial tissues, and vascular and lymphatic elements contribute to beneath the surface structure of the ovary. The germinal epithelium covering the well developed ovary is a continuous layer made up of cuboidal or low columnar epithelial cells resting on a distinct basement membrane. The oogonia in the ovary divide mitotically during the pre-natal life of the individual. This mitotic activity gradually decline and

finally cease to exist just before or soon after birth. The oocytes incapable of mitotic division enter into dictyate phase and are enveloped in ovarian follicles during their growth and maturation. While the majority of oocytes undergo atresia at varying times in the course of their development those destined to survive undergo a series of changes before maturation. Oocytes surrounded by a single layer of flattened epithelial cells are numerous in the adult females and these account for about 90% of the total oocyte population. As they increase in size the follicles gradually sink deeper into the cortex of the ovary and the single layer of flattened cells enveloping the oocyte increases in thickness and cells become cuboidal or columnar to follicles at this stage. Various stages in the development of small oocytes to complete graafian follicles are found in the cortex.

Like thetis, ovary is dual in function i.e., gametogenesis and hormone production. The ovarian activity during adult reproductive life is controlled by reciprocal hormonal action between the ovary and the anterior pituitary gland. The general consensus is that three anterior pituitary hormones FSH, LH and LTH control the structure and function of adult ovary. Except for the earliest phases the follicular growth is under the control of follicle stimulating hormones.

The luteinising hormone brings about ovulation and corpus luteum formation while the luteotrophic hormone influences the secretion of progesterone from corpus luteum. It is also claimed that LH stimulates the secretion of estrogen.

The ovary is known to produce estrogen, progestrone, androgen and relaxin. The first three are the ovarian steroids taking important role in the regulation of reproductive physiology. It is generally assumed that the gonodotrophic activity of the pituitary is itself related by a feedback mechanism to the concentration of ovarian hormones via hypothalamus which is supposed to possess FSH releasing and LH releasing factors.

The mammalian ovary is very sensitive to several physico-chemical agents. Ovaries of certain species of mammals readily respond to the deleterious action of polycyclic aromatic hydrocarbons some of which are potent carcinogens prevailing ubiquitously in human environment. Several strains of mice develop ovarian tumours on exposure to DMBA.

A reduction in the number of follicles is already seen, one month after the treatment with this chemical carcinogen (Marchant, 1957) and no normal follicles are seldom seen after the appearance of tumour nodules in the ovaries (Kuwahara, 1967). The number of small oocytes

was reduced within one or two weeks after the application of a chemical carcinogen regardless of whether the carcinogen was given by mouth, or intraperitonealy or painted directly on to the ovaries (Krarup, 1967). It was suggested that the early destruction of oocytes plays an important role in the subsequent development of ovarian tumours. This is evidenced strongly by the observation that a genetically early deletion of oocytes invariably results in ovarian tumours (Russell and Fekete, 1958; Murphy and Russell, 1963).

It is obvious that an endocrine tumour like that of the ovary must be involved definitely in hormonal regulation and interaction. And the concept that ovarian cancer is definitely due to a hormonal imbalance in the system is repeatedly evidenced by various experts in the field (W.V. Gardner, 1953; Kuwahara, 1967; Hannah Peters, 1969; and Griffith et. al., 1963).

There are many reports of inhibition of ovarian carcinoma by different hormones. Ovarian carcinoma is inhibited by Testosterone (Pels', 1958), Progesterone (Iglerias et. al., 1959) and cortisone (Hardones et. al., 1956). But the mechanism of action of these hormones on the carcinoma is not explained fully.

In addition to the systemic effect by the way of pituitary there are strong indications that estrogens can exert direct local effects upon the ovary (Broadburg, 1951). Also it is obvious that growth hormone plays an important role in many disorders. So in the present investigation insulin and estrogen are taken to find out their influence on the oocytic depletion of the ovary. And it will reveal an idea of the mechanism of action and interaction of these hormones indirectly in ovarian cancer.

MATERIALS AND METHODS

Mice of the Swiss albino strain were used for the present work. They were obtained from Haryana Agricultural University, Hissar. The mice were maintained in the metal boxes in the air conditioned animal-room. They were fed with Hindustan Lever rat feeds available in the form of pellets and water adlibitum. The animals, aging 8 - 10 weeks were chosen for the experiments.

The chemical carcinogen DMBA (7, 12 Dimethyl Benz-anthracene) was selected for the experiments. It was obtained from Eastman Kodac Co., U.S.A. DMBA was dissolved in olive oil (3 mg. in 0.25 ml.) and was given to each experimental animal by intragastric intubation under ether anaesthesia.

Hormones β estradiol (1, 3, 5 (10) - Estrien 3, 17, B diol) and insulin (crystalline) were obtained from Sigma, U.S.A. β estradiol was dissolved in olive oil (100 µg in 0.1 ml.). And insulin was dissolved in physiological saline. Both the hormones β estradiol and insulin were injected sub cutaneously. To avoid the insulin convulsion 0.05 ml. of 10% glucose solution was injected intraperitoneally for every insulin group animal. Totally six experimental groups were set up as mentioned below.

1. The first group of animals were given only olive oil which served as the control group.

2. The second group of animals were given DMBA solution only.

3. The third group of animals were injected with estrogen only.

4. The fourth group of animals were injected with estrogen followed by DMBA.

5. The fifth group of animals were injected with insulin only.

6. The sixth group of animals were injected with insulin for four days continuously and on the fifth day they were given DMBA.

The animals were autopsied at 30 and 45 days after the treatments namely the first and second intervals. The ovaries and uteri were taken and their weights were recorded for statistical analysis. The ovaries were fixed in Bouins solution for about 15 - 20 hours. After the dehydration by graded alcohols they were

embedded in parablast paraffin wax. Serial sections of the ovaries at 5 μ were taken. They were stained with Harris haematoxylin and Eosin for histological and histopathological observations. The differential oocyte counts were performed and the ovarian pathology was studied. The oocytes were divided into the following types.

1. Primordial follicles: The follicles or small oocytes which are surrounded by one or two flat cells.

2. Primary follicles: The follicles which are surrounded by only few cells forming an outer layer.

3. Secondary follicles: The follicles surrounded by more than one cell layer.

4. Tertiary follicles: Follicles with a single antrum or many antra in between the surrounding cell layers.

5. Graafian follicles: Completely formed follicles with a big antrum and other fully formed structures like cumulus oophorus, corona radiata, zona pellucida, theca interna, theca externa etc.

However, like the method followed by Krarup (1969) the primodial follicle type was considered as small oocytes and that the rest of the types as growing and large oocytes. The oocytes

were counted in every 10th section using the nucleolus as the marker. The total number of oocytes in one ovary was calculated by the method described by Peters and Levy (1964) and Krarup (1969) as given below. There was no overcounting and there was no need to use abercombies correction factor since the size of the marker nucleolus and the thickness of the section were the same (5 μ). The number of small oocytes was therefore determined as

The number counted x 10.

Though Jones (1957) found no difference between the two ovaries of several strains of mice oocyte counts were performed on both ovaries of all mice as the pathological development some times differed between them.

RESULTS

Control Groups-

The number of oocytes discussed below in all the groups are the mean number in them. The mean number of small oocytes in the first interval of 30 days after vehicle treatment was 2590 (Table I). And 21.3% of growing and large oocytes to the total number of oocytes were seen in this interval. While in second interval of 45 days after the vehicle treatment some small oocytes were found to be eliminated. This depletion was about 8.5% (Table II) from the small oocytes of the first interval, whereas the percentage of growing and large oocytes to the total number of oocyte population was 23.9%. This is an increase of about 2.6% from the first interval growing and large oocytes' percentage. And there was an increase of 6.4% of growing and large oocytes population from the first interval to the second interval (Histograms I and II).

The ovaries of both the first interval and the second interval were filled with oocytes and follicles (Figs 1,2,3). Small oocytes were found in groups and nests at the periphery of the ovaries (Fig.2). Growing and large oocytes in varying stages of follicle development from the primordial follicle to the complete graafian follicle were

distributed through out most of the organ (Fig.3). Blood vessels in the periovarian capsule and capillaries in the outer ovarian cortex were occasionally noted. Some amount of stroma was present in both the intervals apparently originating from degenerating and degenerated follicles. Large fresh corpora lutea as well as older and smaller corpora lutea were present. Degenerating follicles as well as completely atretic follicles with a remnant of Zonapellucida were like wise found. The second interval ovaries showed more stroma compared to the first interval.

Table II shows the mean weights (± standard deviation) of ovaries and uteri of different groups respectively. The mean weight of ovaries in the first interval of the control group was 4.84 m.gms. In the second interval there was an increase of weight to 19.42% the weight being 5.78 m.gms (Histogram III and IV). The uterus weight showed an increase of 5.78% from the first interval to the second interval. So the above mentioned results show the normal oocytic number, histology and the weights of uteri and ovaries in control.

TABLE I-A

No.	Group	Interval after the treatment	No. of animals	No. of ovaries screened for oocyte counting	No. of small oocytes in: Left ovary	No. of small oocytes in: Right ovary	Total No. of small oocytes	No. of growing and large oocytes in: Left ovary	No. of growing and large oocytes in: Right ovary	Total No. of growing and large oocytes	Total No. of oocytes	Growing and large oocytes / Total oocytes (per animal) x100
1.	Control	30 days	5	4	1410	1300	2710	300	320	620	3330	
					1260	1210	2470	370	410	780	3250	
						Average:	2590		Average:	700		21.3%
		45 days	5	4	1000	1220	2220	350	330	680	2900	
					1260	1260	2520	370	440	810	3330	
						Average:	2370		Average:	745		23.9%
2.	DMBA	30 days	5	6	1020	1250	2270	490	420	910	3180	
					810	990	1800	350	370	720	2520	
					680	770	1450	380	370	750	1520	
						Average:	1840		Average:	793		30.11%
		45 days	5	4	510	520	1030	400	290	690	1720	
					710	630	1340	250	260	510	1850	
						Average:	1185		Average:	600		33.61%

TABLE I-B

No.	Group	Interval after the treatment	No. of animals	No. of ovaries screened for oocyte counting	No. of small oocytes in: Left ovary	No. of small oocytes in: Right ovary	Total No. of small oocytes	No. of growing and large oocytes in: Left ovary	No. of growing and large oocytes in: Right ovary	Total No. of growing and large oocytes	Total No. of oocytes	Growing and large oocytes / Total oocytes (per animal) x 100
3.	Estradiol	30 days	5	6	1300	1360	2660	410	330	740	3400	
					1250	1190	2440	350	310	660	3100	
					1140	970	2110	310	390	700	2810	
						Average:	2403		Average:	700		22.56%
		45 days	5	4	1390	1130	2520	290	340	630	3150	
					1070	960	2030	440	440	880	2910	
						Average:	2275		Average:	755		24.92%
4.	DMBA + Estradiol	30 days	5	8	1120	1050	2170	260	320	580	2750	
					1190	1170	2360	270	240	510	2870	
					1320	1260	2580	300	280	580	3160	
					940	1120	2060	300	320	620	2680	
						Average:	2292*		Average:	573		20%
		45 days	5	4	1370	880	2250	330	390	720	2970	
					1110	1680	1790	320	330	650	2440	
						Average:	2020+		Average:	685		25.32%

* = Significantly different with 1st interval of DMBA group ($P < 0.2$); Non-significantly different with 1st interval of control group.

\+ = Significantly different with 2nd interval of DMBA group ($P < 0.2$); Non-significantly different with 2nd interval of control group.

TABLE I-C

No.	Group	Interval after the treatment	No. of animals	No. of ovaries screened for oocyte counting	No. of small oocytes in: Left ovary	Right ovary	Total No. of small oocytes	No. of growing and large oocytes in: Left ovary	Right ovary	Total No. of growing and large oocytes	Total No. of oocytes	Growing and large oocytes / Total oocytes (per animal) x100
5.	Insulin	30 days	5	4	1200	1070	2270	320	380	700	2970	
					1330	1340	2670	370	350	720	3390	
						Average:	2470		Average:	710		22.33%
		45 days	5	4	1200	1080	2280	390	440	830	3110	
					1010	1230	2240	410	380	790	3030	
						Average:	2260		Average:	810		26.39%
6.	DMBA + Insulin	30 days	5	6	730	680	1410	370	260	630	2040	
					790	790	1580	350	360	710	2290	
					750	780	1530	320	350	670	2200	
						Average:	1507		Average:	670		30.78%
		45 days	5	4	640	660	1300	370	340	710	2010	
					640	650	1290	320	330	650	1940	
						Average:	1295		Average:	680		34.43%

TABLE II

Mean weights (in m.gms.) of ovaries and uteri in the control and experimental groups.

No.	Groups	Interval (Days after the treatment)	Ovaries (in pair) Mean ± S.D.*	Uteri Mean ± S.D.*
1.	Control	30	4.84 ± 0.6	46.02 ± 4.46
		45	5.78 ± 0.64	48.68 ± 3.37
2.	DMBA	30	2.8 ± 0.346	33.2 ± 2.79
		45	2.42 ± 0.46	27.78 ± 2.80
3.	Estradiol	30	5.88 ± 0.628	52.8 ± 2.47
		45	5.5 ± 0.47	61.26 ± 3.8
4.	DMBA + Estradiol	30	4.62 ± 0.6	36.68 ± 4.4
		45	5.16 ± 0.45	30.6 ± 6.3
5.	Insulin	30	5.94 ± 0.725	50.12 ± 6.7
		45	5.02 ± 0.245	53.38 ± 4.4
6.	DMBA + Insulin	30	3.14 ± 0.608	36.26 ± 3.66
		45	3.84 ± 0.82	35.4 ± 3.68

* = Standard Deviation.

TABLE III

Comparison of oocytes in the different groups

No.	Group	Interval	Comparison with Group	Comparison with Interval	Percentage difference for Small oocytes	Percentage difference for Growing & large oocytes
1.	Control	30 days	Control	45 days	*<8.49%	●>6.43%
2.	DMBA	30 days	Control	30 days	<28.96%	>13.3%
3.	DMBA	30 days	DMBA	45 days	<35.6 %	<24.34%
4.	DMBA	45 days	Control	45 days	<50%	<19.46%
5.	Estradiol	30 days	Estradiol	45 days	<5.33%	>7.86%
6.	DMBA + Estradiol	30 days	Estradiol	30 days	<4.62%	<18.14%
7.	DMBA + Estradiol	30 days	DMBA + Estradiol	45 days	<11.86%	>19.55%
8.	DMBA + Estradiol	45 days	Estradiol	45 days	<11.21%	<9.27%
9.	Insulin	30 days	Insulin	45 days	<8.5%	>14.08%
10.	DMBA + Insulin	30 days	Insulin	30 days	<38.99%	<5.63%
11.	DMBA + Insulin	30 days	DMBA + Insulin	45 days	<5.63%	<14.06%
12.	DMBA + Insulin	45 days	Insulin	45 days	<42.7%	<16.05%

* < = Less

● > = More

TABLE IV

Comparison of ovarian and uterine weights in the different groups.

No.	Group	Interval	Comparison with Group	Comparison with Interval	Percentage difference for Ovarian weights	Percentage difference for Uterine weights
1.	Control	30 days	Control	45 days	*<19.42%	⊙>5.78%
2.	DMBA	30 days	Control	30 days	*<42.15%	<27.86%
3.	DMBA	30 days	DMBA	45 days	<13.57%	<16.33%
4.	DMBA	45 days	Control	45 days	<58.13%	<42.9%
5.	Estradiol	30 days	Estradiol	45 days	<6.47%	>16.02%
6.	DMBA + Estradiol	30 days	Estradiol	30 days	<21.43%	<30.53%
7.	DMBA + Estradiol	30 days	DMBA + Estradiol	45 days	>11.69%	<16.58%
8.	DMBA + Estradiol	45 days	Estradiol	45 days	<6.18%	<50.05%
9.	Insulin	30 days	Insulin	45 days	<15.49%	>6.51%
10.	DMBA + Insulin	30 days	Insulin	30 days	<47.14%	<27.65%
11.	DMBA + Insulin	30 days	DMBA + Insulin	45 days	>22.3%	<2.37%
12.	DMBA + Insulin	45 days	Insulin	45 days	<23.51%	<33.68%

* < = Less

⊙ > = More

DMBA Group:-

This group showed remarkable changes in the oocytic number as well as in ovarian histology.

In the first interval the small oocytes were about 1840 (Table IA). It is a sudden depletion to about 28.96% from the control group. In the second interval the small oocytes further decreased to 1185 a further decrease of 35.6% from the first interval (Table III). The percentage of second interval is very high when compared to the control group (8.5%). So it can be said that the effect of DMBA is continuous in the second interval also.

The growing and large oocytes were 793 during the first interval. It shows an increase of 13.29% from the first interval of the control group. In the second interval the growing and large oocytes decreased to 600, the decrease being 24.34% from the first interval. Whereas in the control group there was an increase of growing and large oocytes to 6.43% from the first interval to the second interval. And in this group the percentage of growing and large oocytes to the total number of oocytes was 30.1% in the first interval and 33.6% in the second interval.

Their difference between the two intervals is 3.5% whereas it was only 2.6% in the control group.

The ovarian histology of the first and second interval showed lot of degenerative and pathological changes (Figs.4&6). The ovaries were smaller than the control and it was prominent in the second interval.

Periovarian hyperaemia and dilation of capillaries in the outer cortical layer were seen in the first interval ovaries. Oocytes of all sizes were seen but spaces in the sub-epithelial layer apparently left by degenerated small oocytes were characteristic (Figs.5&7). Follicle degeneration was marked in both the intervals.

In the second interval the surface epithelium was cuboidal and often double. Large amounts of stroma with areas of lutenisation were characteristic. The lutenised stroma was apparently derived from degenerative follicular material. Occasionally empty rings and pseudo follicles could be seen in the outer cortex lying between the follicles and the corpora lutea. (Figs.5&7). Corpora lutea was prominent and in some ovaries an accumulation of old corpora lutea seemed to be present and the limit between the adjacent corpora lutea

was sometimes ill defined. The amount of stroma was large. Some degenerating follicles as well as many atretic follicles with a remnant of zona pellucida or hyalinized ovum were present. In a few of large follicles with a degenerate ova, lutenization appeared to be taking place to form 'corpora lutea atretica'.

In the second interval the germinal epithelium was very thick and dense. The germinal epithelial cells seemed to be condensed and prominent as in irradiated ovaries by other authors (Hannah Peters, 1969; June Marchant, 1957). Histologically there was a mild involution of ovarian cortex. Many hyalinized scars denoting degenerated follicles could be seen all over the cortex.

The mean weight of the ovaries was 2.8 m.gms. in the first interval. It is a decrease of 42.15% from the first interval of control group (Table II). The second interval ovarian weight further decreased for about 13.57% from the first interval (Table IV), the mean weight being 2.42 m.gms. It is to be noted that the percentage of increase of ovarian weight in the control olive oil group from the first interval to the second interval was only 19.42%. So the effect of DMBA is seen both in the first interval as well as in the second interval, by the decrease in the ovarian weights. The

uterine weights also decreased from the control group, the weights being 33.2 m.gms. in the first interval and 27.78 in the second interval. The weight decrease in the ovaries and uteri may be attributed to the pathological development by DMBA in them.

Estradiol Group:

The estradiol group serves as the control for the study of DMBA + Estradiol group. The total number of small oocytes in the first interval was 2403 and 2275 in the second interval. Obviously it does not show much difference when compared to the control group. From the first interval to the second interval the depletion of oocytes was 5.33% (Table III), which is not significantly different with the control group. The growing and large oocytes were 700 in the first interval and there was a 7.86% increase from this in the second interval. The percentage of growing and large oocytes to the total number of oocytes indicates an increase of 2.5% from the first interval to the second interval.

The ovaries of this group were more larger than the other groups with increase of weights. There was no significant pathological changes and so there was no much difference from the control group. Overall the ovary was healthy with normal follicles, some-

times even with bigger follicles (Figs.8&9). Corpora lutea were big. 3/4th of the ovary was usually filled up by the corpora lutea and sometimes the boundary between two corpora lutea was not seen. The stroma was loose and so there were lot of spaces in between them. And the number of atretic follicles and empty rings was very minimal.

The weight of the ovaries showed a sharp increase to about 5.88 mg. in the Ist interval and 5.50 mg. in the second interval. The decrease of weight in the second interval may be attributed to the diminishing effect of the hormone with the gradual time elapse. On the other hand uteri also showed the increase of weight showing the influence of the hormone but the weight increased during the second interval than the first. It shows indirectly the late effect of the hormone on the uterus.

DMBA + Estradiol Group:

When compared with the former DMBA group and estradiol group this group definitely shows that estradiol protects the ovaries from DMBA's action. Because, though the carcinogen DMBA had been given, this group did not show much pathological changes either by oocytic depletion or by ovarian pathology.

The small oocytes were about 2292 in the first interval (Histogram I). When compared to the estradiol group the depletion of oocytes by DMBA in the presence of estradiol was 4.62%. But in the case of DMBA group the depletion from the control group was 28.96%. This shows that the oocytic depletion by DMBA was almost blocked by estradiol. The second interval small oocytes showed a decrease of 11.86% from the first interval, whereas it was 35.60% in DMBA group. So it is obvious that the DMBA's action is completely reduced by estradiol. The growing and large oocytes were 573 in the first interval which is about 20% in the total number of oocytes. During the second interval it increased to 25.3%. It shows that the DMBA action on the growing and large oocytes is more in the first interval when compared to the second interval. It is to be noted that the increase of the growing and large oocytes to the total oocyte population from the first interval to the second interval was more in this group than that of the estradiol group.

When compared to the DMBA group here estradiol has decreased the percentage of growing and large oocytes in the first interval. And the increase from the first interval to the second interval is more in the presence of estradiol (Table IB. ; Histogram II).

The histopathology of this group did not show the prominent DMBA actions when compared with estradiol and DMBA groups. The germinal epithelium was normal like the control group in both the intervals (Fig.11). The atretic follicles were minimal and so the sub-epithelial spaces were very few. The small oocytes were mostly healthy as in the control group and almost all the follicle types were present (Fig.11). The graafian follicles were large with all its accessory structures like theca interna, theca externa, cumulus cophorus etc. The stroma formed by the atretic follicles are more in the second interval than in the first. The corpora lutea were very large. So there were some pathological effect of DMBA in the presence of estradiol. It is very minimal when compared with the effect of DMBA alone. It is obvious from this to conclude that estradiol gives some protection to the ovaries from the pathological action of DMBA.

The mean weight of the ovaries in the first interval of this group was 4.62 m.gms (Table II). It is a decreased weight when compared to the weight of estradiol group which shows indirectly the action of DMBA on the ovary. The decrease of the ovarian weight of this group to the estradiol group is found to be 21.43% whereas

the same decrease between control and DMBA group was 42.15%. So it is obvious from these percentages that the action of DMBA is affected by estradiol at least to about 50%. In the second interval the ovarian weight has increased to about 11.69%, whereas in DMBA group there was a decrease in the ovarian weight to about 13.6%. So it can be concluded that the effect of DMBA was completely washed out by estradiol when the ovary was taken out in the second interval. The uterus showed a decreased weight when compared to control and estradiol groups. The difference of decrease between estradiol and this group in the first interval was 30.5% whereas it was only 27.86% in the case of control and DMBA groups. So there was an increased action of DMBA on uterus than in control. From this it can be assumed that the action of DMBA on ovary is diverted to uterus by estradiol. In the second interval the uterus weight decreased from the first interval to about 16.6%, the exact mean weight being 30.6 m.gms. The decrease was 5.78% in the case of control group (Table IV). So even in this second interval the action of DMBA is more on uterus in the presence of estradiol.

Insulin Group:

In this insulin group the number of small oocytes did not show any peculiar counting other than the control and estradiol groups. In the first interval, the small oocytes were 2470. So it did not differ much with control group which had 2590 small oocytes (Table I-C). The second interval had decreased small oocytes than the first interval which was 2260. The decrease from the first interval was 8.5% which falls in line with the control group that had 8.49% depletion (Table III).

The growing and large oocytes counting also did not show much difference with the control group. In this group the growing and large oocytes were 710 in the first interval and 810 in the second interval, the increase in the later interval being about 14.08%. When compared with the control group there was no difference in the first interval but in the second interval the increase of growing and large oocytes was 7% more in this group. It may be attributed to the late action of insulin to push the small oocytes towards the growing side.

The histology of the insulin group did not show much difference from the control group. The germinal epithelium and its

cells were normal. The ground cells or the granulosa cells were found to be slightly more than the other groups. The corpora lutea were normal and their number was very minimal. The ovary did not show any space in the sub-epithelial region or in medulla. Very few but normal graafian follicles were seen. Among the growing and large oocytes, follicles surrounded by two layers of cells were abundant in the group.

The mean weight of the ovaries of this group was 5.94 mg. in the first interval. The second interval weight was 5.02 mg. which was 6.5% less than the first interval (Table II). In the first interval the ovarian weight is more than the control group whereas in the second interval it is lesser. It shows the sudden action of insulin to increase the weight of the ovaries in the first interval and the effect being slowly decreasing after the time elapse.

The mean weight of the uterus was 50.12 mg. in the first interval an increase of 4 m.gm. than the control. It may be attributed to the action of insulin. In the second interval the uterus weight further increased to 53.38 m.gm. the increase being 6.5% from the first interval. This second interval increase was 5.78%

than the control group. So it can be said that in the second interval also there was a mild action of insulin to increase the uterine weights.

DMBA + Insulin Group:

This group shows the oocytic depletion as in the case of DMBA group. In the first interval the small oocytes' number was 1507. It is a decrease of 39% from the insulin group's Ist interval whereas in the control and DMBA groups the decrease was 28.96% (Table III). So it shows that the depleting action of DMBA on the oocytes was more in the presence of insulin. In the second interval the small oocytes were 1295. It is a decrease of 14.06% from the first interval. But in the case of insulin group it was only 8.5% which shows the persisting depleting action of DMBA in the second interval also. But however in the control group the decreasing difference in the second interval was 35.6%. So from this it is very clear that in the presence of insulin the DMBA's action was more concentrated in the Ist interval than in the second interval. But whereas in the DMBA group it was distributed in both the intervals.

There was no much difference between the first and the second interval growing and large oocytes in number, the average being 1507 in the former and 1295 in the latter. In the first interval there was a decrease of growing and large oocytes to about 5.63% when compared to the insulin group. This decrease is very less when compared with the former DMBA and estradiol groups. From this it is obvious that the DMBA does not have any remarkable effect on the growing and large oocytes in the presence of insulin.

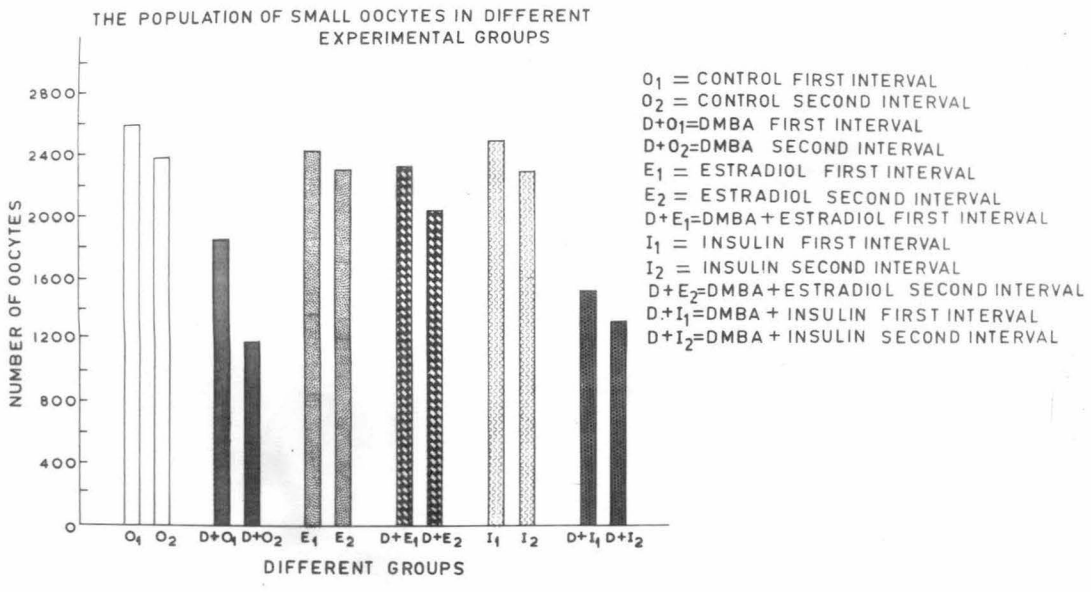
The histology of the ovary of this group showed slight pathological effect of DMBA. The germinal epithelium was normal. Sometimes empty rings and atretic follicles were seen. Different stages of growing and large oocytes filled the ovaries completely (Fig. 14.). Among the growing and large oocytes the antral follicles were found to be more. Since the stroma was not compact, there were number of spaces in the medullary region of the ovaries.

The ovarian mean weight in the first interval of this group was 3.14 m.gms. which is a decrease of 42.15% from the insulin group. The second interval showed 22.3% increase in the weight - the weight being 3.84 m.gm. It means that the DMBA's action of decreasing the ovarian weight is over by the first interval. When

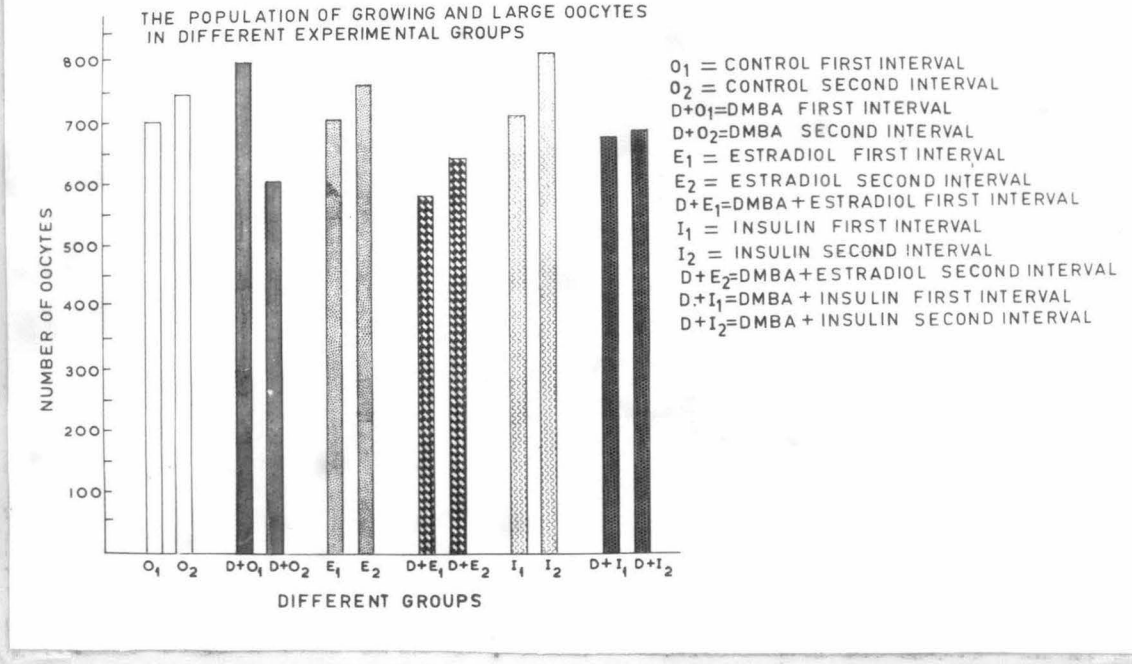
compared to the DMBA group the weight of the ovaries is more. It shows that insulin increases the ovarian weight in the presence of DMBA some how. It is possible that DMBA's action on the ovaries is obstructed to some extent by the presence of insulin.

The mean weight of the uteri was 36.26 m.gms. in the first interval. When compared to the insulin group the weight has decreased to 27.65%. So there is no much difference in the DMBA's action on ovaries by the presence of insulin. The weight of the uterus decreased to 35.4 m.gms. in the second interval - the decrease being about 2.37% only. But this decrease in the control and DMBA group was 5.78% and 16.33% respectively. So from this it is understood that the long run action of DMBA on the uterus is affected in the presence of insulin.

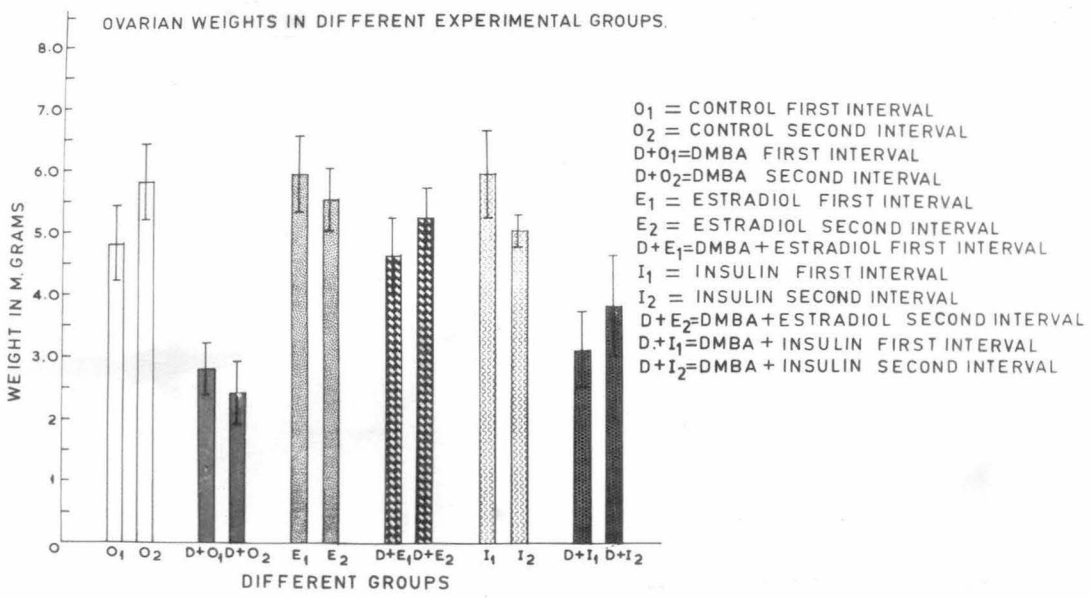
Histogram I


THE POPULATION OF SMALL OOCYTES IN DIFFERENT EXPERIMENTAL GROUPS
NUMBER OF OOCYTES
2800
2400
2000
1600
1200
800
400
0
O1 O2 D+O1 D+O2 E1 E2 D+E1 D+E2 I1 I2 D+I1 D+I2
DIFFERENT GROUPS
O1 = CONTROL FIRST INTERVAL
O2 = CONTROL SECOND INTERVAL
D+O1=DMBA FIRST INTERVAL
D+O2=DMBA SECOND INTERVAL
E1 = ESTRADIOL FIRST INTERVAL
E2 = ESTRADIOL SECOND INTERVAL
D+E1=DMBA+ESTRADIOL FIRST INTERVAL
I1 = INSULIN FIRST INTERVAL
I2 = INSULIN SECOND INTERVAL
D+E2=DMBA+ESTRADIOL SECOND INTERVAL
D+I1=DMBA+INSULIN FIRST INTERVAL
D+I2=DMBA+INSULIN SECOND INTERVAL

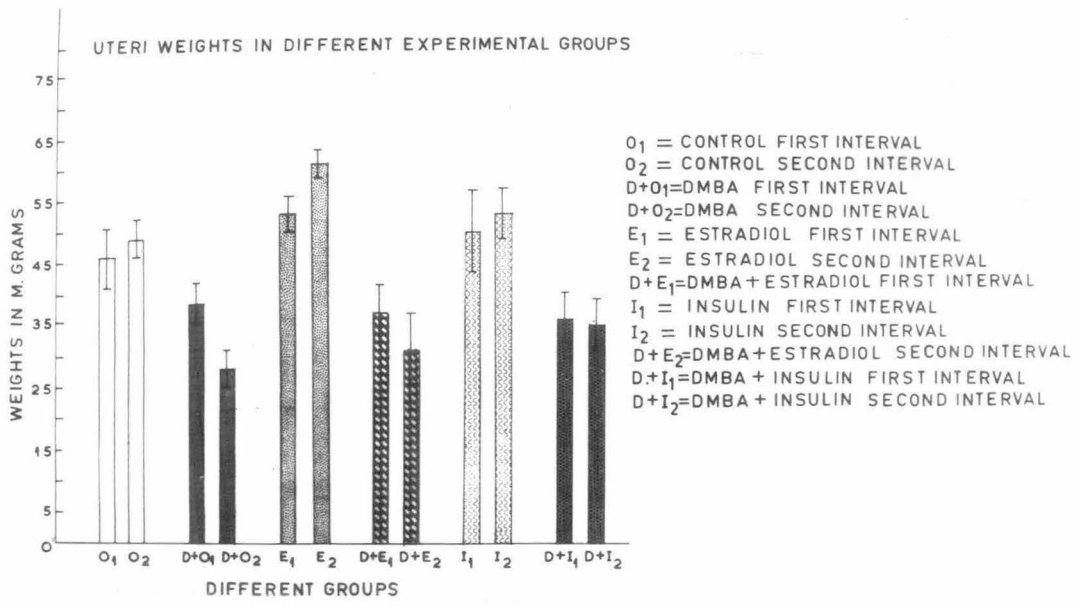
Histogram II



Histogram III



Histogram IV



DISCUSSION

Present series of experiments were designed to see the effect of polycyclic aromatic hydrocarbon-DMBA on the ovary of mice and the modulation of this effect by administration of exogenous hormones like estrogen and insulin.

Our preliminary studies have already indicated that olive oil which has been used as a vehicle for DMBA administration does not elicit any adverse effects on the ovaries at the given dose level. Krarup *et. al.* (1969) also have shown that olive oil treated animals do not deviate in their oocytic number or ovarian histology from those of untreated animals.

DMBA is a potent carcinogen and it elicits tumorigenesis in organs like mamary glands, ovaries, adrenal gland, kidney, testes etc. DMBA needs transformation before it triggers neo - plastic transformation in the target tissues or organs. The liver is known to take active part in this biotransformation process. However other organs and tissues containing appropriate enzyme system also bring this kind of biotransformation. The pathological changes seen in the ovary of mice in the present experiment should have been brought about by DMBA or its metabolites formed locally

in the ovary. Because topical application of DMBA on the ovary can also elicit such changes (Krarup et. al., 1969).

DMBA given by intragastric intubation to the animals affected the small oocytes of the ovaries and reduced their number. The oocyte destruction was very high in the period of 30 days as well as in 45 days after the treatment. According to Krarup (1969) in the case of DMBA painted directly on the ovary, the oocytic depletion was limited to the first four weeks after the treatment. After this time the small oocytes were eliminated at normal rate (Krarup, 1969). So there is less destruction of oocytes by oral ingestion of DMBA than by direct application on the ovary. The growing and large oocytes seem to be unaffected by DMBA. Though they are found to be decreased in the second interval of the DMBA group the percentage to the total number of oocytes shows an increase i.e. 30.12% in the first interval and 33.62% in the second interval. So it can be understood that the reduction in the number of growing and large oocytes is definitely secondary to the reduction in the number of small oocytes. The relative resistance of larger follicles might possibly related to

changes in the oocyte prior to meiosis and ovulation or to changes in follicular function with more advanced development. It may be pertinant to that, the smaller follicles do not require pituitary stimulation for growth upto 100 to 200 μ in diameter whereas larger follicles are dependent on FSH and LH.

Concurrently with the process of germ cell elimination pathological changes develop in the ovaries. It has been suggested that the neoplastic development is secondary to the premature elimination of oocytes and not caused by the carcinogen itself (Krarup, 1964). This is supported by the observation that ovarian tumour invariably develops following the genetic deletion of germ cells (Russell and Fakete, 1958; Murphy and Russell, 1963), and that, among the four strains of mice spontaneous ovarian tumours only occured in that particular strain whose ovaries were physiologically exhausted of oocytes within the life span of the animals (Jones and Krohn, 1961).

According to Pederson and Krarup (1969) there is an immediate effect on the granulosa cells by DMBA which accelerates

the follicle growth rate. When this stimulating effect has subsided, the remaining follicles continue to develop normally.

The ovarian pathology showed many atretic granulosa cells in DMBA group when compared to the control group. Besides degeneration of oocytes the early post treatment changes include the appearance of empty rings and pseudofollicles. These characteristic structures have been noted by several authors after X-irradiation and described as anovular follicles (Guthrie, M.I., 1958; William G. Slate, 1962; Srivastava, P.N. and Ramesha Rao, A., 1968). Their origin is unclear and had been ascribed to (a) remnants of small follicles in which the oocytes have degenerated or (b) differentiation of embryonal cells lying dormant in the ovarian stroma or (c) formation from the germinal (surface) epithelium (Thung et. al., 1956).

In the present study empty rings and pseudofollicles have not been observed to be connected with the surface epithelium. That they are formed from follicles whose oocytes have degenerated is unlikely, because oocytes in follicles which have a size comparable to pseudofollicles (i.e. type 3 and type 4 follicles; Pedarson

and Peters, 1968) are not destroyed by DMBA. Their number decreases because the pool of small oocytes from which they are recruited is reduced (Krarup, 1969 b; Krarup et. al., 1969). It is therefore most likely, that empty rings and pseudofollicles have been formed from cells belonging to ovarian stroma. In the immature mouse ovary, follicle cells are known to derive from stroma cells (Peters and Pederson, 1967) and it is possible that such cells may differentiate to follicle like structures under these experimental condition where oocytes are absent.

Diffuse lutenized tissue derived from confluent corpora lutea and lutenized stroma and its peripheral collection of pseudo-follicles were found in the ovaries of animals treated with DMBA. It is one of the important preneoplastic changes noted. It represents the end point of the initiation phase of ovarian tumour (Marchant, 1961; Howell J.S. et. al., 1954). The ovaries of DMBA group suffered an enormous percentage of weight decrease. The uteri weight also decreased considerably in the DMBA group than the control group. It is known already that estrogen administration leads to the increase of RNA and protein synthesis in

the uterus, and there by increasing the weight of it. So it is possible to assume here that DMBA decrease the quantity of estrogen in the system. Because of this action there was less RNA and protein synthesis thereby reducing the weight of the uterus.

The results of control, DMBA, estradiol and DMBA + Estradiol groups show that estradiol somehow protects the ovaries from the DMBA's actions. There was no significant pathological action of DMBA in the presence of estradiol by oocyte number or by ovarian pathology and uterine and ovarian weights.

Exogenous estradiol definitely interacts with the ovarian functions combining with the nuclei of granulosa cells. It was evidenced by Stumpf, W.E. (1969) with the results that ^{3}H estradiol bound strongly to the nuclei of the cells of uterus, vagina, and to the nuclei of the granulosa cells of the ovary. There are many evidences indicating that the exogenous estradiol exerts a strong inhibitory effect on the secretion of LH and FSH (Vernon L. Gay et. al., 1970; Davidson J.M. et. al., 1970; Ajika et. al., 1972; Swerdloff and Wash, 1973; Zainisi and Martini, 1975;

Nillius and Wide, 1975 and D.R. London & R.W. Shaw, 1978). So it is evident that the external estradiol given might have affected the pituitary FSH and LH secretion. The decrease in the estrogen level by DMBA was evidenced by the low weight of uterus in the DMBA treated animals. So DMBA's action should be through the sex hormone estrogen.

It is possible that DMBA by reducing estrogen in the system stimulates the pituitary secretions FSH and LH which in turn depletes the small oocytes. But however Marchant, J. (1961) proved categorically that pituitary factors did not involve in the initiation phase of ovarian tumour (oocytic depletion) by DMBA. Her conclusion was based on the observation that preneoplastic changes including depletion of oocytes and follicles readily developed after DMBA application in hypophysectemized animals while the further tumour development only occured in the presence of the pituitary. So though it is tempting to correlate the LH and FSH involvement in the depletion of oocytes through estrogen, it cannot be, unless Marchant's experiment is repeated and disproved.

So with the present results and previous literature it is possible to correlate DMBA's action on the ovary of depleting the oocytes and further development of ovarian tumour as follows:

Initiation Phase (DMBA-the initiating factor)	Promotion Phase (Pituitary-the promoting factor)
1. DMBA-Decrease the estrogen level in the ovary; particularly in the granulosa cell environment. ↓	
2. Depletion of oocytes and pathological changes in the ovary. ↓	4. More and more FSH and LH from pituitary. ↓
3. Estrogen level decreasing very much.	5. Ovarian tumour.

So it is possible that estrogen decrease by DMBA is equalised by the exogenous estrogen given with DMBA and because of it the depletion of oocytes by DMBA is obstructed in the DMBA + Estradiol group.

Although insulin is not considered as a primary hormone in tumour growth recent studies have shown that alterations in the insulin status of the host or of the medium invitro resulted in altered growth of the tumours. Insulin was required in the culture

medium where the tumour system was studied invitro. This has been reviewed by Topper (1970). Data obtained invitro indicated that insulin stimulated DNA synthesis and that the wave of DNA synthesis was essential for further differentiation of the explant. If insulin is essential for DNA synthesis invivo, tumour growth or tumorigenesis may be influenced signi - ficantly by the host's insulin status.

Heuson and his colleagues (Heuson, J.C. and Legros, 1970; Heuson, J.C. and Legros, 1971; Heuson, J.C., Legros and Hermann, 1972) studied the role of insulin in induction of mammary tumours by DMBA, as well as the role of insulin in subsequent tumour growth. Insulin was shown to stimulate DNA synthesis in DMBA induced tumours organ culture invitro. About 90% of these carci- nogen induced tumours regressed invivo after inducing diabetes in tumour bearing animals by alloxan treatment (Cora P. Cherry and Glucksmann, 1971). When insulin was administered concomitantly with DMBA to sprague-Dawley rats, the tumour incidence paradoxi- cally reduced (Rao, 1977). So with these ideas insulin was tried to see its effect on the oocytic depletion of the ovary. From the results it is understood that insulin enhanced the action of

DMBA some how in the first interval. It was evidenced by the major depletion of oocytes, decrease in the weight of ovaries and uteri etc. But in the second interval there were less amount of depletion of oocytes and increase in the weights of uteri and ovaries. So it is found that insulin concentrated DMBA's action to the first interval and in the second interval it reduced the depletion of oocytes while increasing the weights of ovaries and uteri.

Being an endocrine tumour the ovarian neoplasm might be definitely due to a kind of hormonal imbalance (Robert A. Huseby, 1965). So apart from these estrogen and insulin, experiments with other related hormones like LH, FSH, prolactin, progesterone etc. will give a clear picture of the hormonal interaction in the tumour which will positively lead to a rational endocrine therapy for it.

SUMMARY

1. The present investigation deals with the influence of hormones like estrogen and insulin on the preneoplastic changes elicited by DMBA in the ovaries of Swiss Albino Mice.

2. Six experimental groups were set up as follows:

i) Control Group; ii) DMBA Group; iii) Estradiol Group; iv) DMBA + Estradiol Group; v) Insulin Group; & vi) DMBA + Insulin Group.

The ovaries of them were taken in two intervals (30 and 45 days after the treatment) and were studied for their oocytic number and pathology.

3. DMBA was given by intragastric intubation. Progressive depletion of small oocytes in both the intervals was seen by the effect of DMBA. But the depletion was restricted to the first four weeks after the treatment when the DMBA was applied directly on the ovary (Krarup, 1969).

4. The most important finding from our investigation is that the oocytic depletion and the pathological changes by DMBA were reduced by Estradiol. So it is suggested that DMBA's action

should be through suppression of estrogen level in the ovary.

5. Insulin did not give any significant results. It neither significantly increased nor significantly decreased the effect of DMBA.

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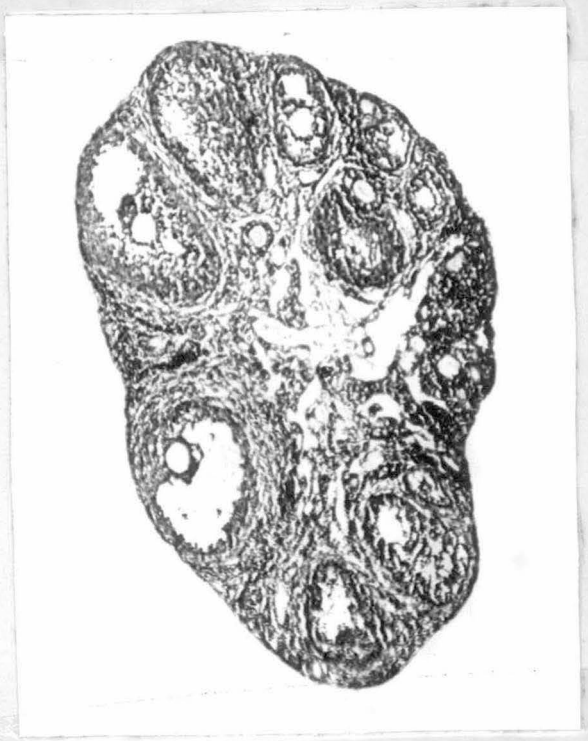
Explanation of the photomicrographs

1. A section from the ovary of control group Ist interval showing the normal ovarian structure (x 80)

2. A section from the control group Ist interval showing the normal and healthy small oocytes(x 640)

1



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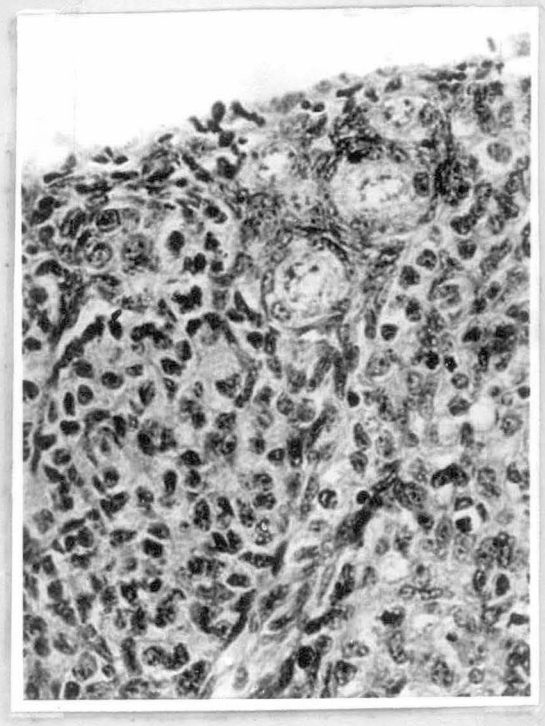
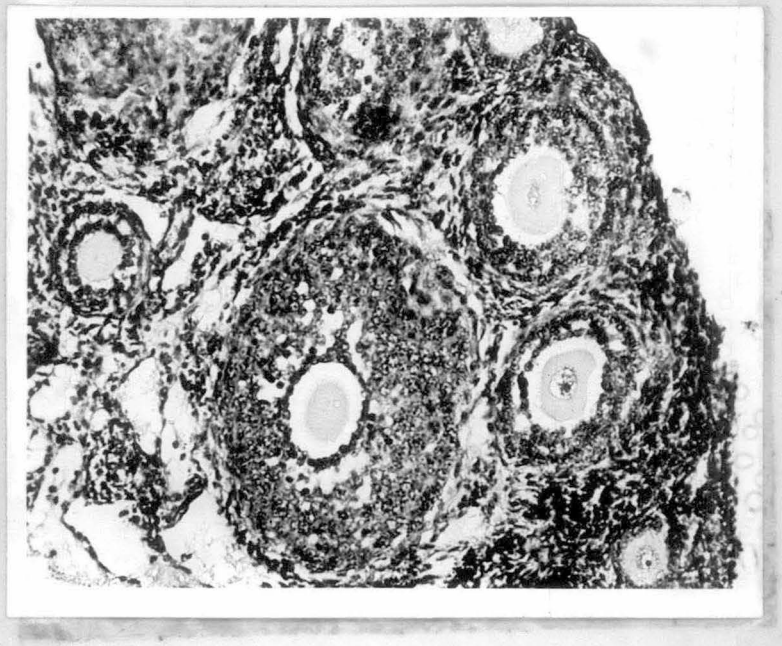


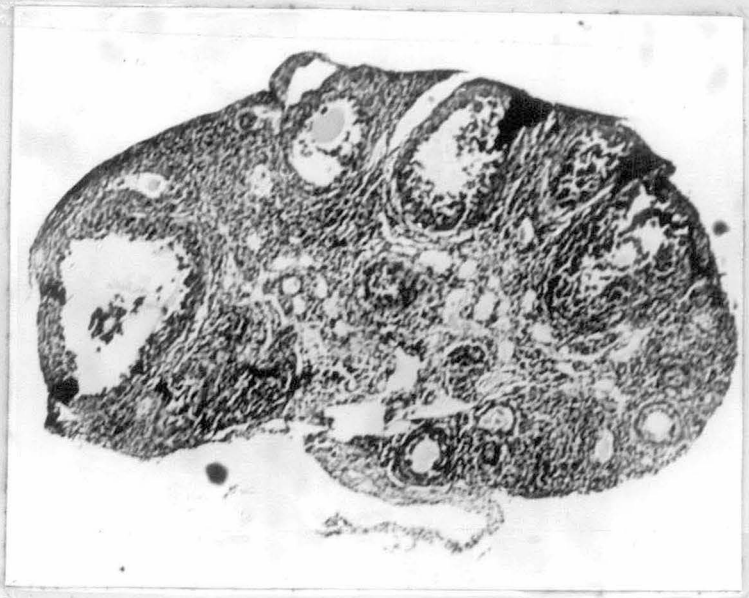
3. An ovarian section from the control group Ist interval showing the normal and healthy growing oocytes (x 640)

4. A section of ovary from DMBA group Ist interval showing the empty rings and atretic follicles (x 100)

3



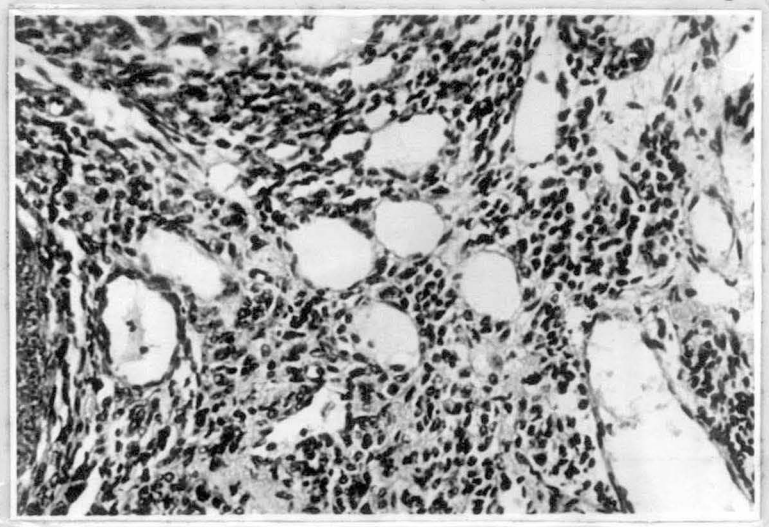
4



5. A magnified part of an ovarian section from DMBA group Ist interval showing the empty rings (x 320)

6. An ovarian section from DMBA group IInd interval showing a number of atretic follicles and empty rings (x 100)

5



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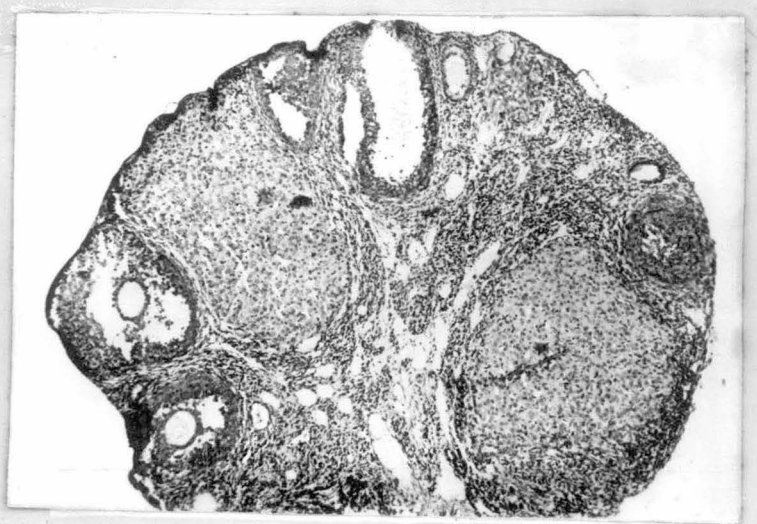
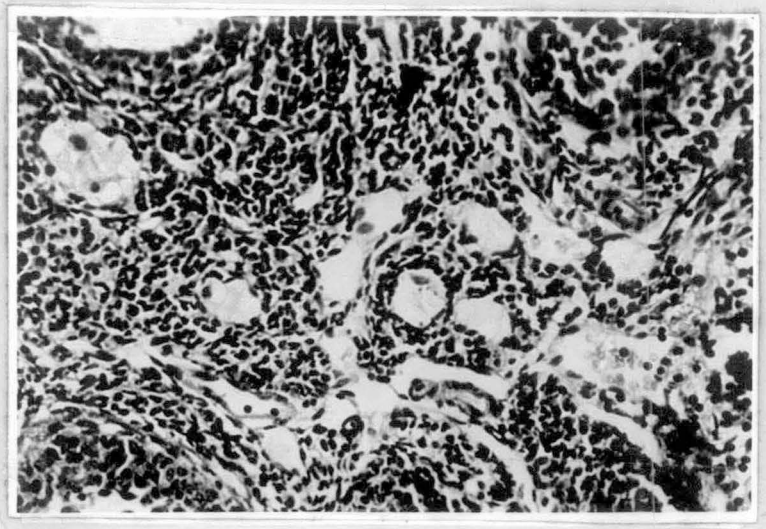


7. A magnified part of an ovarian section from DMBA group IInd interval showing the empty rings (x 320)

8. An ovarian section from Estradiol group Ist interval showing the healthy ovarian structures (x 80)

7



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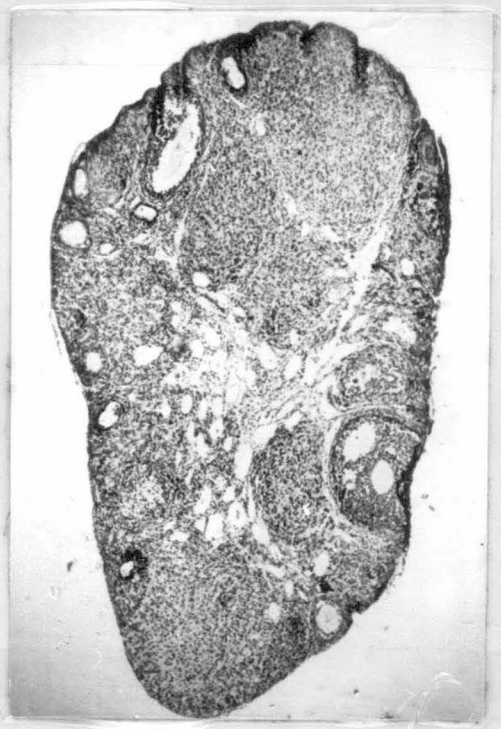
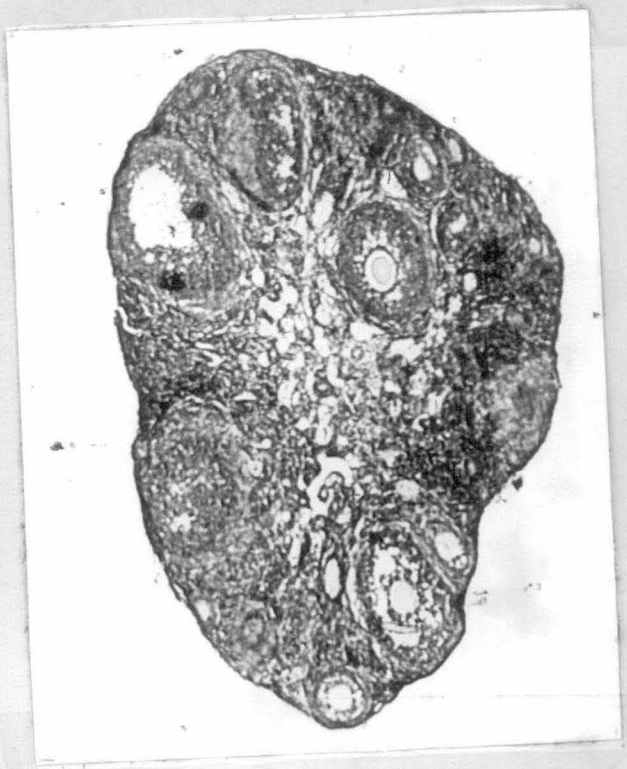


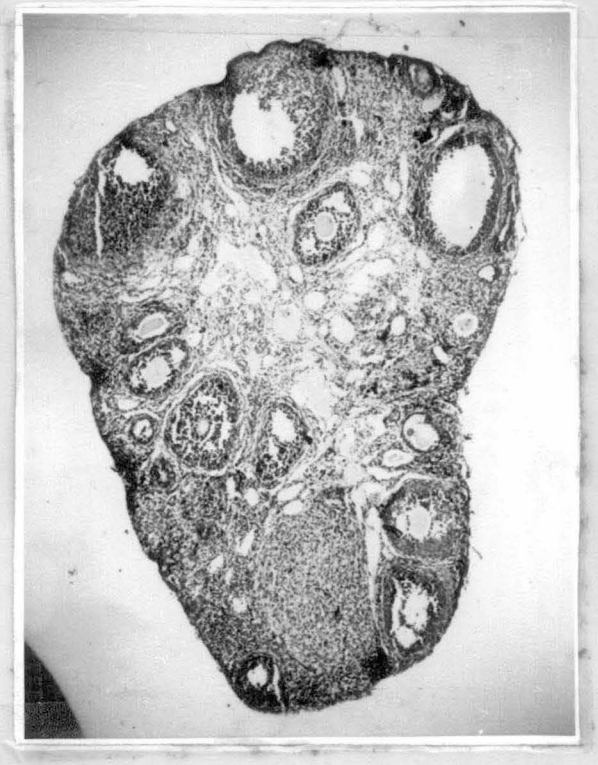
9. A section of ovary from the Estradiol group IInd interval showing the healthy ovarian components (x 80)

10. A section of ovary from DMBA + Estradiol group Ist interval showing the healthy oocytes (x 80)

9



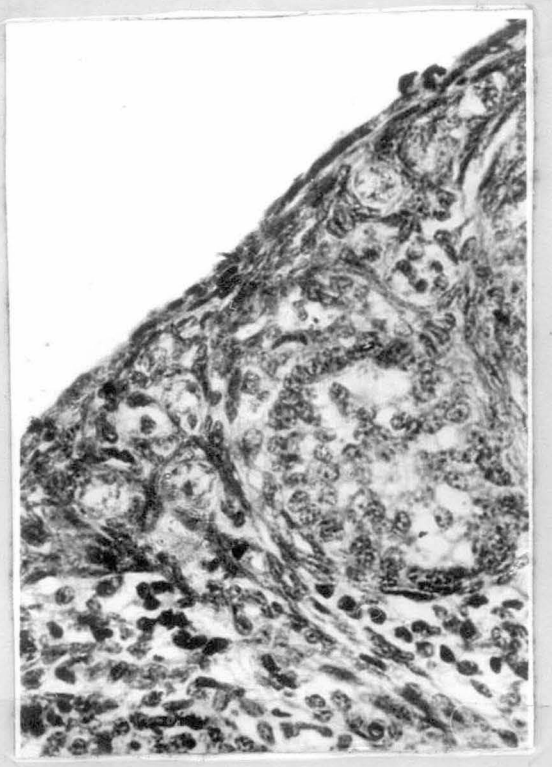
10



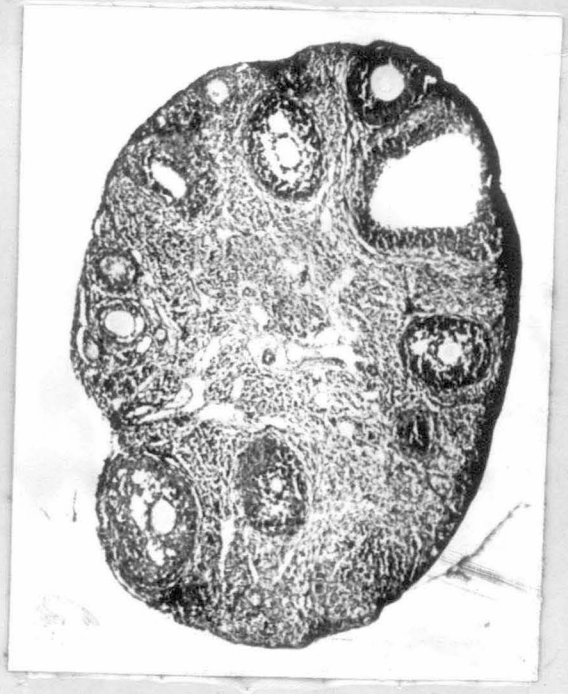
11. A magnified part of the previous ovarian section from DMBA + Estradiol group IInd interval showing the healthy small oocytes (x 640)

12. A section from the ovary of Insulin group Ist interval showing the healthy structures (x 80)

11



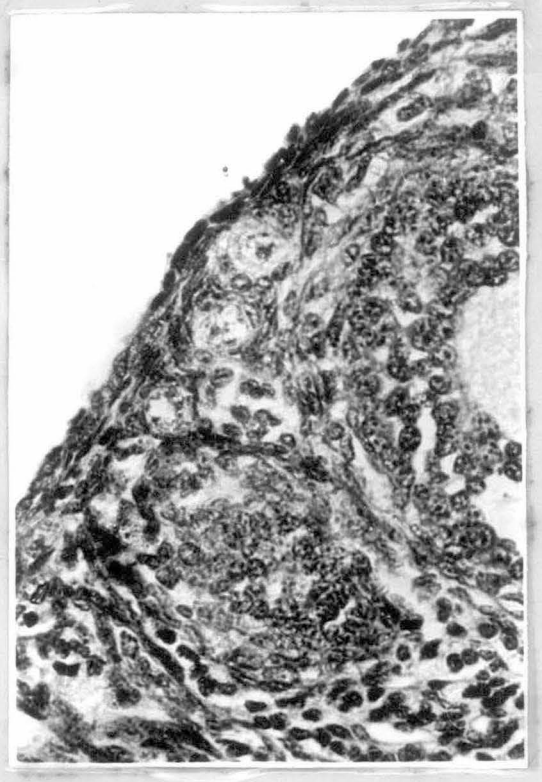
12



13. An ovarian section from the Insulin group IInd interval showing the healthy small oocytes (x 64)

14. A section from DMBA + Insulin group Ist interval showing the empty rings and atretic follicles (x 80)

13



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