INFLUENCE OF BHA (A FOOD ADDITIVE) ON OOCYTIC DEPLETION INDUCED BY DMBA (A CARCINOGEN) IN MICE

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CERTIFICATE

The research work embodied in this M.Phil. dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067. The work is original and has not been submitted so far, in part or full, for any other degree or diploma of any University or Institute.

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CONTENTS

INTRODUCTION

As our understanding of the causes of various types of cancer unfolds, it is becoming apparent that a minority of cancers are genetically fated to appear, while most are caused by environmental factors.

Higginson (1969) has suggested that the lowest reported cancer incidences represent natural levels and that any increase from the baseline values generally can be attributed to environmental influences. According to Higginson (1969) 90% human cancer incidences can be ascribed to environmental factors. Boyland (1967) is more specific in that he concluded that chemical components are responsible for 90% of human cancer development and 10% is caused by genetic, viral and radiation factors. Thus, in the sixties oncologists redirected, to a significant extent, their research focus on environmental factors from the man-made chemical carcinogens and radiant energy to naturally occuring chemical compounds which may be present in human food.

Industrialization, urbanization and transportation continue to pour a significant number of these chemical carcinogens into the environment. It is quite natural the

human beings are bound to be exposed to those agents and subsequently prone to get cancer. Henceforth, survival of human beings is in part a function of their ability to either prevent the intrusion of toxic foreign chemicals from the environment or to remove them by detoxification mechanisms. The metabolism of exogenous chemicals is a major determinant of their ultimate biological activity. The enzymatic conversion of the chemical carcinogens may yield less harmful derivatives but it may also cause the formation of active intermediates that are either toxic or carcinogenic (Gelboin et al., 1969, 1972 and Miller, 1970).

As the information concerning the chemistry and metabolism of chemical carcinogens and biochemistry of cancer in general has been increasing, and when cancer cure is a remote possibility, one should certainly probe into its preventive aspects. Thus, in seventies oncologists started focussing their attention to a greater extent on agents which inhibit the action of carcinogens, although experiments on the inhibition of chemical carcinogenesis date back to 1929. At that time it was shown empirically that dichloro ethyl sulfide inhibits skin tumour formation

resulting from repeated paintings of mouse skin with carcinogenic tar (Berenblum, 1929).

There are many chemical compounds that have the capacity to modify or inhibit the effect of chemical carcinogens with low toxicity administered either prior to or after the exposure of the carcinogens. The following are the potential mechanisms of modification of chemical carcinogenesis:

i) reversal of early carcinogenic process,

ii) alteration of the metabolism of the carcinogen,

a) decreased activation,

b) increased detoxification,

c) combination of (a) and (b),

iii) scavanging of active molecular species of carcinogens to prevent them from reaching critical target sites in the cells,

iv) competitive inhibition.

Overall the modification entiles the prevention of active form of the carcinogen from reaching or reacting with the target sites.

A number of naturally occurring organic compounds having the capacity to inhibit the neoplastic effects of chemical carcinogens have been identified. These are:

coumarins and some related simple lactones, phenols, organic isothiocyanates, indoles, and flavones. Coumarins occur in many plants. The parent compound, coumarin, is a moderately potent inhibitor of polycyclic aromatic hydrocarbon (PAH) carcinogens whereas two commonly occuring derivatives, 7-hydroxycoumarin (umbelliferone) and 7-hydroxy 6-methoxycoumarin (scopoletin) have thus far shown either weak or no inhibitory activity (Feur and Kellen, 1974; Wattenberg and Leong, 1970). Another naturally occuring lactone, α-angelicalactone inhibits the neoplastic effects of PAH carcinogens (Wattenberg, 1978). Unfortunately there are many natural carcinogens in food and carcinogens can also be formed during the storing or cooking of food (Miller et al., 1980). Hence, searching for synthetic compounds for their inhibitory action on carcinogenesis needs attention.

Recent advancement in food technology has resulted in processing of human food items by using agents or additives which serve as preservatives, anti-oxidants, emulsifiers, thickners, buffers, flavour or colour enhancers and even as nutrient supplements. At present processed food items contain more than 2,500 different additives (Reif, 1981). Since 1972, a Committee of Federation of American Societies

for Experimental Biology (FASEB) has reviewed some of the 400 substances classified as "generally recognized as safe" (GRAS). As a result a number of these substances have been placed on an interim regulated list for further evaluation.

As the enzymatic activation of carcinogens involves oxidation (Wattenberg, 1978), the use of antioxidants as possible inhibitors of chemical carcinogens is based, in general, on the concept that the anti-oxidants will exert a seavanging effect on the reactive species of carcinogen, thus protecting cellular constituents from attack. Also, according to Handler (1979), apart from inhibiting oxidation of carcinogens the modifiers destroy free radicals such as superoxides (O_2^-) and OH-radical which can induce mutation in DNA and bring about uncontrolled proliferation of cells.

In earlier studies, wheatgerm oil and α-tocopherol were employed. Experiments showing positive and negative results have been documented and have been summarised by Wattenberg (1972). During the past several years studies carried out with other anti-oxidants have shown to inhibit the effects of substantial variety of chemical carcinogens.

The most extensive work of this type has been done with phenolic antioxidants, butylated hydroxyanisole (BHA)

and butylated hydroxytoluene (BHT), despite their enlistment in the interim regulated list. Inhibitory modulation occurs in a variety of experimental conditions and with a broad range of chemical carcinogens (Wattenburg, 1978). Some thiols, phenols, amines and quinolines are also anti-carcinogenic although they may not possess toxicities as low as those of BHA and BHT. Also several non phenolic anti-oxidants, for example, ethoxyquin, a commercial food additive was shown to have inhibitory action on carcinogenic and toxic effects of PAH, when tested in lung, fore stomach and breast (Wattenberg, 1972), were found to inhibit chemical carcinogenesis.

Experiments on the capacity of disulphiram and some related compounds to inhibit chemical carcinogenesis have been done. These sulfur-containing compounds are potent inhibitors of benzo(a)pyrene (BP) induced neoplasia of the forestomach and large intestine (Wattenberg, 1976). When added to the diet disulphiram and diethyl dithiocarbonate profoundly inhibit large bowelneoplasia (Wattenberg, 1975). Inhibition of chemical carcinogenesis by selenium salt has also been reported. Sodium selenide added to the croton oil suppressed the development of skin tumour (Shamberger, 1966). In a subsequent work methylcholantherene (MCA) was

repeatedly applied to the skin and the addition of sodium selenide inhibited epidermal neoplasia (Shamberger, 1977).

Several studies have demonstrated that protection against chemical carcinogens by the administration of inducers of increased microsomal mixed function oxidase activity, is possible. The inducers employed varied from compounds such as polycyclic aromatic hydrocarbons which are noxious agents, to chemicals such as flavones which have little toxicity (Wattenberg & Leong, 1970).

As noted earlier BHA is one of the phenolic anti-oxidants - a group of compounds widely used in the food consumed by human population (Chipault, 1966). It has been found in situations where the route of administration results in direct contact of carcinogen with the target tissue (ie) occurrence of neoplasia of forestomach in mice fed BP or 7, 12-dimethylbenzanthracene (DMBA) (Wattenberg, 1972) It was found when rats were fed with PAH carcinogen containing diets, most of them developed gastric neoplasia. But when BHA or BHT added to the diet, the incidence was significantly lessened. Comparative suppression of neoplasia was also obtained in experiments in which the carcinogen is acting at a site remote from that of administration. An example is the inhibition of mammary tumour formation in rats given DMBA orally.

BHA and BHT are of primary interest because of their extensive use as food additives. Of the two compounds BHA is preferable, since it is less toxic than BHT. However, both of them can be employed at very high doses before the evidence of toxicity appears (Hathaway, 1966).

When BHA and BHT, at a concentration of 5 mg/g diet were added to BP (1 mg/g diet) containing diet, inhibition of carcinogenesis at the forestomach of mouse was noticed (Wattenberg, 1972). The human consumption of these phenolic anti-oxidants is of the order of the magnitude of several milligrams a day. Assuming that the results of experiments on animals hold good for man, this amount of antioxidants could be of importance in inhibiting the effects of chronic exposure to low doses of carcinogens, the type of exposure which is most likely to occur in human population.

The exact mechanism by which the antioxidants inhibit neoplasia has not been established and may differ for various antioxidants. Several possibilities exist which can be divided into two major categories. The first involves some type of direct interaction between anti-oxidant and reactive species of the carcinogen. The second possibility is that the antioxidant may positively modulate the

detoxifying enzyme apparatus. According to Wattenberg (1975) BHA acts by increasing cytochrome P450 in the liver and by altering the microsomal mixed function oxidase system.

BHA decreases the epoxidation (which is an activation process) and increases the detoxification (i.e. production of 3-hydroxybenzo(a)pyrene) of PAH (Lam and Wattenberg, 1977). Further, BHA elicits a marked increase in glutathione-S-transferase activity in mice (Benson et al., 1979). BHA is also known to adversely affect the binding of carcinogenic metabolites to DNA (Speier and Wattenberg, 1975; Speier et al., 1978).

Although BHA has been used in checking experimental carcinogenesis in different organs, its influence on ovarian carcinogenesis induced by PAH is not at all known. Hence, present investigation is related to the chemical interactions with the early events taking place in the ovary, prior to the tumour development.

Ovary is a well defined entity consisting of a variety of different cell types. It has a well defined morphological and functional interrelationship and thus exhibits 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.

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 germinal epithelium underneath which lies, the tunica albuginea, a connective tissue layer. Follicular apparatuses, stromal and connective tissues, interstitial tissues, and vascular and lymphatic elements contribute 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. Thus mitotic activity gradually declines and finally cease to exist just before or soon after birth. Oocytes which are 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 a single layer of flattened cells enveloping the oocyte increases in thickness and cells become cuboidal or columnar follicles at this stage. Various stages in the development of small oocytes to complete graafian follicles are formed in the cortex.

Like testis, the ovary plays a dual role in mammalian reproduction: it produces the hormones necessary for establishing the appropriate internal milieu for pregnancy and it nurtures the oocyte which carries genetic information required to form a new individual. The ovarian activity during the adult reproductive life is controlled by reciprocal hormonal action between the ovary and the anterior pituitory gland. The general consensus is that three anterior pituitory hormones FSH, LH and LTH control the structure and function of the ovary. Except for the earliest phases, the follicular growth is under the control of follicle stimulating hormones. The luteinizing 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 linked by a feedback mechanism to the concentration of ovarian hormones via hypothalamous which is supposed to possess FSH releasing and LH releasing factors.

Exposure of a female to xenoboitic compounds may impair reproduction by interfering with either facet of ovarian function. Of greater concern, however, are those compounds that interact with oocytes, as those interactions may have profound effects on the latent genetic message stored in the gametes. The study of oocyte-xenoboitic interactions becomes even more compelling with the realization that oogenesis, the formation of new oocytes, does not occur after birth in most mammals (Zuckerman and Baker, 1977).

Ovaries of certain species of mammals readily respond to the deleterious action of polycyclic aromatic hydrocarbons (PAH), 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 seen one month after the treatment (Marchant, 1957) and no normal follicles are 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 through mouth, or intraperitonealy or painted directly on the ovaries (Kraup, 1967). It is evidenced that pre-treatment of animals with pregnant mare serum gonadotropin (PMSG) enhanced DMBA induced oocyte depletion and increased DMBA activity in the ovaries (Rao, 1981). It has been established that PAH brings oocytic depletion in different strains of mice (Mattison, 1979). It has also been shown that PAH produce meiotic errors in oocytes, i.e. chromosome aberrations in oocytes of mice (Basler and Rohiborn, 1976). The early destruction of oocytes plays an important role in the subsequent development of ovarian tumours. This is evidenced strongly by the observation that an early genetic deletion of oocytes invariably results in ovarian tumours (Russell and Fakete, 1968; Murphy and Russell, 1963; Kraup, 1969, 1970a, 1970b; Murphy, 1972; Jull, 1973). Also the concept that ovarian cancer im definitely due to a hormonal

imbalance in the system is repeatedly confirmed by various researchers in the field (Gardner, 1953; Kuwahara, 1967; Hannah Petey, 1969; and Griffith et al., 1963). And it is not fully known how PAH brings hormonal imbalance and thereby causes ovarian cancer. Recently, it has been documented that estrogen offers protection against destruction of oocytes by DMBA (Manoharan and Rao, 1980).

As already stated depletion of oocytes is a pre-requisite for the development of ovarian tumours, and DMBA, a potent carcinogen, is known to elicit oocytic depletion in the ovary of mice. In the present study an attempt has been made to see whether different doses of BHA given concomitantly with DMBA to the virgin female mice would check the depletion of oocytes by DMBA. If this modulator inhibits the depopulation of oocytes, the chances of ovary developing tumours should also be reduced, and thus this method could turn out to be a good prophylactic means to control chemical carcinogenesis in the ovary.

MATERIALS AND METHODS

ANIMALS:

Ramdombred Swiss albino mice were used in these experiments. Young adult female mice (6-8 week old) obtained from the Experimental Animal facility, All India Institute of Medical Sciences, New Delhi, were maintained in plastic cages with rice husk lining at 25 ± 2°C. They were fed with standard rat feed (Hindustan Lever Ltd., India) and water ad libitum.

CHEMICALS:

The chemical carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) obtained from Eastman Kodak Co., USA was dissolved in olive oil to yield a concentration of 12 mg carcinogen in 1 ml oil.

The food-additive butylated hydroxyanisole (BHA) in powder form was procured from Sigma Chemical Company, U.S.A. It was mixed with finely powdered rat feed (Hindustan Lever Ltd., India) to attain two dose levels (i.e. 5 mg/g diet and 10 mg/g diet) and the mixture was stirred for about 30 minutes to ensure proper mixing. The "experimental diets" were prepared freshly once in two days.

Experimental design:

The animals were assorted into the following control and experimental groups:

Group - I: Animals (14 Nos.) of this group were administered with only olive oil by intragastric intubation to serve as controls. They were maintained in normal diet.

Group - II: Animals (16 Nos.) of this group were given DMBA in olive oil (3 mg/0.25 ml) by intragastric intubation and maintained in normal diet.

Group - III: Animals (12 Nos.) of this group were fed with the experimental diet containing BHA 5 mg/g diet only for about two weeks and then maintained in normal diet.

Group - IV: Animals (16 Nos.) of this group were given DMBA (3 mg/0.25 ml/mouse) after being on the experimental diet containing BHA 5 mg/g diet for ten days. The experimental diet was continued for 3 more days after the administration of carcinogen.

Group - V: Animals (12 Nos.) of this group were fed with the experimental diet containing BHA 10 mg/g diet only as in Group III.

GROUP - VI: Animals (16 Nos.) of this group were given DMBA (3 mg/0.25 ml/mouse) after being on the experimental diet containing BHA 10 mg/g diet as in Group-IV.

The animals were autopsied at 30th and 45th days after the treatments. The ovaries and uteri were removed and their weights recorded for statistical analysis. The ovaries were fixed in Bouin's fluid for about 20-24 hrs. After the dehydration by alcohol in upgrade series they were embedded in 'paraplast' paraffin wax (melting point 58-60°C). Serial sections of the ovaries were taken at 5 μ thickness. They were stained with Harris Haematoxylin and Eosin for histological and histopathological observations. Differential oocyte counts were performed and in addition ovarian pathology was studied. The oocytes were classified into the following types:

i) Primordial follicles - Small oocytes which are surrounded by 2-3 flat cells.

ii) Primary follicles - The follicles which are surrounded by a single layer of cuboidal cells.

iii) Secondary follicles - The follicles surrounded by more than one layer of cells.

iv) Tertiary follicles - Follicles with a single antrum or many antra in between the surrounding cell layers.

v) Graafian follicles - Completely formed follicles with a big antrum and other fully formed structures like cumulus oophorous, corona radiata, zona pallucida, theca interna, theca externa etc.

However, as per the method followed by Kraup (1969), the primordial follicle types were considered as small oocytes as growing and large oocytes. Using nucleoli of the oocytes as markers, the viable oocytes were counted in every 10th section. The total number of oocytes in one ovary was calculated according to Peters and Levy (1964) and Kraup (1969). There was no over counting 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 (8 μ). The number of small oocytes was, therefore, determined as:

the number counted x 10.

Though Jones and Krohn (1961) 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 as recorded by Manoharan and Rao (1980).

RESULTS

Group - I (CONTROL)

In all cases the number of oocytes is represented as mean ± SD. The mean number of small oocytes (SO), growing and large oocytes (GLO) and total number of oocytes per animal are depicted in Table I. Out of the oocytes present in the ovaries of mice, 30 days after vehicle treatment, 78% form SO whereas 22% constitute GLO. At 45th day, SO decreased by about 15% and GLO increased by about 12% (Table IIa, Fig. I) and these differences were found to be insignificant. Total number of oocytes showed mild decrease during the follow up period, which is attributable to spontaneous atresia.

The ovaries at first interval (30th day after treatment) and second interval (45th day after treatment) filled with oocytes and follicles. SO were found in groups and nests at the periphery of the ovaries. GLO, in varying stages of follicular development, were distributed throughout the organ. Large fresh corpora lutea as well as older and smaller corpora lutea were noticed. Degenerating follicles as well as completely

OOCYTE COUNTS IN DIFFERENT GROUPS

TABLE I(a)

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 Mean ± S D	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 Mean ± S D	Total No. of Oocytes Mean ± SD	Growing and large Oocytes / Total Oocytes (per animal) × 100
1	2	3	4	5	6	7	8	9	10	11	12
CONTROL	30 days	6	4	2980	1890	3960	540	490	1030	4990	
				1870	1590	3460	620	460	1080	4540	
I					Average	3710		Average	1055	4765	22.14 %
						± 353.55			± 35.36	± 318.19	
	45 days	6	4	1700	1610	3310	580	550	1130	4440	
				1200	1810	3010	630	610	1240	4250	
					Average	3160		Average	1185	4345	27.27
						± 212.13			± 77.78	± 134.35	
DEBA	30 days	6	4	460	320	780	420	320	740	1520	
				480	510	990	490	430	920	1910	
II					Average	885		Average	830	1715	48.40%
						± 148.49			± 127.28	±275.77	
	45 days	6	4	160	280	440	280	310	590	1030	
				370	340	710	290	270	560	1270	
					Average	575		Average	575	1150	50.00%
						± 190.92			± 21.21	± 169.71	

TABLE I(b)

1	2	3	4	5	6	7	8	9	10	11	12
DMA	30 days	6	4	19306	1870	3800	530	490	1820	4920	
5 mg/ g diet				1680	1880	3560	510	520	1030	4590	
											21.79%
					Average	3680		Average	1025	4705	
XII						± 169.71			± 7.07	± 162.63	
	45 days	6	4	1550	1450	3000	640	610	1250	4250	
				1740	1910	3650	560	590	1150	4800	
											26.52%
					Average	3325		Average	1200	4525	
						± 459.62			± 70.71	± 388.91	
DMBA+ DMA 5 mg/ g diet	30 days	6	6	1340	1420	2760	610	500	1110	3870	
				1020	750	1770	580	620	1200	2970	
				920	790	1710	570	600	1170	2880	
											35.80%
IV					Average	2080		Average	1160	3240	
						± 589.00			± 45.82	± 547.45	
	45 days	6	4	650	450	1080	570	280	650	1730	
				960	740	1700	440	390	830	2530	34.7 %
					Average	1390		Average	740	2130	
						± 438.41			± 127.28	±569.69	

TABLE I(c)

1	2	3	4	5	6	7	8	9	10	11	12
BBA 10 mg/ g diet	30 days	6	4	2170	2070	4240	550	520	1070	5310	
				1970	1850	3800	520	480	1000	4800	20.47%
					Average	4020		Average	1035	5055	
V						± 311.13		Average	± 49.50	± 360.62	
	45 days	6	4	1700	1880	3580	540	630	1170	4750	
				1590	1980	3570	610	580	1190	4760	24.82%
					Average	3575		Average	1180	4755	
						± 7.07			± 14.14	± 7.07	
DBBA+ BBA 10 mg/ g diet	30 days	6	6	1480	1380	2860	510	400	910	3770	
				1140	1080	2220	610	560	1170	3390	
				1090	970	2050	490	540	1030	3080	30.38%
VI					Average	2377		Average	1037	3414	
						± 427.12			± 130.13	± 345.59	
	45 days	6	4	600	450	1050	530	410	940	1990	
				580	380	960	540	260	600	1560	
					Average	1005		Average	770	1775	43.38%
						± 63.64			± 240.42	± 304.06	

TABLE II(a)

Comparison of Oocytes in different groups

No.	Group	Interval after the Treatment	Comparison with Group	Interval after the Treatment	Percentage difference for small Oocytes	Level of Significance	Percentage difference for large Oocytes	Level of Significance
1	2	3	4	5	6	7	8	9
1.	Control	45 days	Control	30 days	- 15	*	+ 12	*
2.	DMBA	30 days	Control	30 days	- 76	$t_{0.01}$ = 10.418 ≥ 99%	- 21	*
3.	DMBA	45 days	DMBA	30 days	- 35	*	- 31	*
4.	DMBA	45 days	Control	45 days	- 82	$t_{0.01}$ = 12.809 ≥ 99%	- 51	$t_{0.01}$ = 10.70 ≥ 99%
5.	BBA 5 mg/g diet	45 days	BBA 5 mg/g diet	30 days	- 10	*	+ 17	*
6.	DMBA+ BBA 5 mg/ g diet	30 days	BBA 5 mg/g diet	30 days	- 43	$t_{0.05}$ = 3.567 ≥ 95%	+ 13	*
7.	DMBA+ BBA 5 mg/ g diet	45 days	DMBA+ BBA 5 mg/ g diet	30 days	- 33	*	- 36	$t_{0.05}$ = 5.79 ≥ 95%
8.	DMBA+ BBA 5 mg/ g diet	45 days	BBA 5 mg/g diet	45 days	- 58	$t_{0.05}$ = 4.308 ≥ 95%	- 38	*

* NOT SIGNIFICANT

TABLE II(b)

Comparison of Counts in different groups

1	2	3	4	5	6	7	8	9
9.	DMBA+ BHA 5 mg/g diet	30 days	DMBA	30 days	+135	$t_{0.1}$=2.676 ≥ 90%	+ 40	$t_{0.05}$=4.303 ≥ 95%
10.	DMBA+ BHA 5 mg/g diet	45 days	DMBA	45 days	+141	*	- 29	*
11.	BHA 10 mg/ g diet	45 days	BHA 10 mg/ g diet	30 days	- 11	*	+14	*
12.	DMBA+ BHA 10 mg/ g diet	30 days	BHA 10 mg/ g diet	30 days	- 41	$t_{0.05}$=4.588 ≥ 95%	+ 1	*
13.	DMBA+ BHA 10 mg/ g diet	45 days	DMBA+ BHA 10 mg/ g diet	30 days	- 58	$t_{0.05}$=4.28 ≥ 95%	- 25	*
14.	DMBA+ BHA 10 mg/ g diet	45 days	BHA 10 mg/ g diet	45 days	- 72	$t_{0.001}$=66.76 ≥ 99%	- 35	*
15.	DMBA+ BHA 10 mg/ g diet	30 days	DMBA	30 days	+ 68	$t_{0.05}$=4.53 ≥ 95%	+ 85	*
16.	DMBA+ BHA 10 mg/ g diet	45 days	DMBA	45 days	+ 74	$t_{0.1}$=3.20 ≥ 90%	+ 34	*

* Not Significant

TABLE-III: Mean weights (in mg) of ovaries and uteri in the control and experimental groups.

No.	Groups	Interval (days after the treatment)	Ovaries (in pair) Mean ± SEM	Uteri Mean ± SEM
1	Control	30	11.51 ± 0.65	98.9 ± 16.02
		45	14.98 ± 0.81	100.58± 18.46
2	DMBA	30	13.12 ± 1.85	96.35± 26.85
		45	15.06 ± 0.96	91.74± 8.37
3	BHA 5 mg/g diet	30	12.85 ± 0.88	149.96± 22.80
		45	13.93 ± 1.99	70.20± 8.69
4	DMBA + BHA 5 mg/g diet	30	15.94 ± 1.80	119.46± 13.31
		45	10.87 ± 0.78	88.59± 11.90
5	BHA 10 mg/g diet	30	12.63 ± 1.67	78.70± 15.39
		45	14.04 ± 0.75	96.72± 11.86
6	DMBA + BHA 10 mg/g diet	30	11.94 ± 1.48	67.73± 10.97
		45	10.49 ± 1.02	89.82± 14.88

TABLE IV(a)

Comparison of Ovarian & Uterine Weights in different groups

No.	Group	Interval after the treatment	Comparison with Group	Interval after the treatment	Percentage difference for Ovarian weights	Level of Significance	Percentage difference for Uterine unit weights	Level of Significance
1	2	3	4	5	6	7	8	9
1.	Control	45 days	Control	30 days	+ 30	$t_{0.01}$=3.467 ≥ 99%	+ 2	*
2.	DMBA	30 days	Control	30 days	+ 14	*	- 3	*
3.	DMBA	45 days	DMBA	30 days	+ 15	*	- 5	*
4.	DMBA	45 days	Control	45 days	+ 1	*	- 9	*
5.	BHA 5 mg/ g diet	45 days	BHA 5 mg/ g diet	30 days	+ 8	*	- 53	$t_{0.01}$=3.564 ≥ 99%
6.	DMBA+ BHA 5 mg/ g diet	30 days	BHA 5 mg/ g diet	30 days	+ 24	*	- 28	*
7.	DMBA+ BHA 5 mg/ g diet	45days	DMBA+ BHA 5 mg/ g diet	30 days	- 32	$t_{0.01}$=3.094 ≥99%	- 26	$t_{0.1}$=1.815 ≥ 90%
8.	DMBA+ BHA 5 mg/ g diet	45 days	BHA 5 mg/ g diet	45 days	- 22	$t_{0.01}$=1.824 ≥ 95%	+ 26	*

* NOT SIGNIFICANT

TABLE IV(b)

Comparison of Ovarian & Uterine Weights in different groups

1	2	3	4	5	6	7	8	9
9.	DMBA+ BEA 5 mg/ g diet	30 days	DMBA	30 days	+ 21	*	+ 24	*
10.	DMBA+ BEA 5 mg/ g diet	45 days	DMBA	45 days	- 27	$t_{0.01}$=3.641 ≥ 99%	- 3	*
11.	BEA 10 mg/ g diet	45 days	BEA 10 mg/ g diet	30 days	+ 11	*	+ 23	*
12.	DMBA+ BEA 10 mg/ g diet	30 days	BEA 10 mg/ g diet	30 days	- 5	*	- 14	*
13.	DMBA+ BEA 10 mg/ g diet	45 days	DMBA+ BEA 10 mg/ g diet	30 days	- 12	*	+ 33	*
14.	DMBA+ BEA 10 mg/ g diet	45 days	BEA 10 mg/ g diet	45 days	- 25	$t_{0.05}$=2.768 ≥ 95%	- 7	*
15.	DMBA+ BEA 10 mg/ g diet	30 days	DMBA	30 days	- 9	*	- 30	*
16.	DMBA+ BEA 10 mg/ g diet	45 days	DMBA	45 days	- 30	$t_{0.01}$=3.150 ≥ 99%	- 2	*

* NOT SIGNIFICANT

FIGURE I

O_1	=	Control Group First Interval
O_2	=	Control Group Second Interval
$D+O_1$	=	DMBA Group First Interval
$D+O_2$	=	DMBA Group Second Interval
B_1	=	BHA/5mg/g diet group First Interval
B_2	=	BHA 5 mg/g diet Group Second Interval
$D+B_1$	=	DMBA+BHA 5 mg/g diet First Interval
$D+B_2$	=	DMBA + BHA 5 mg/g diet Second Interval
B_3	=	BHA 10 mg/g diet First Interval
B_4	=	BHA 10 mg/g diet Second Interval
$D+B_3$	=	DMBA + BHA 10 mg/g diet First Interval
$D+B_4$	=	DMBA + BHA 10 mg/g diet Second Interval.

Fig: I

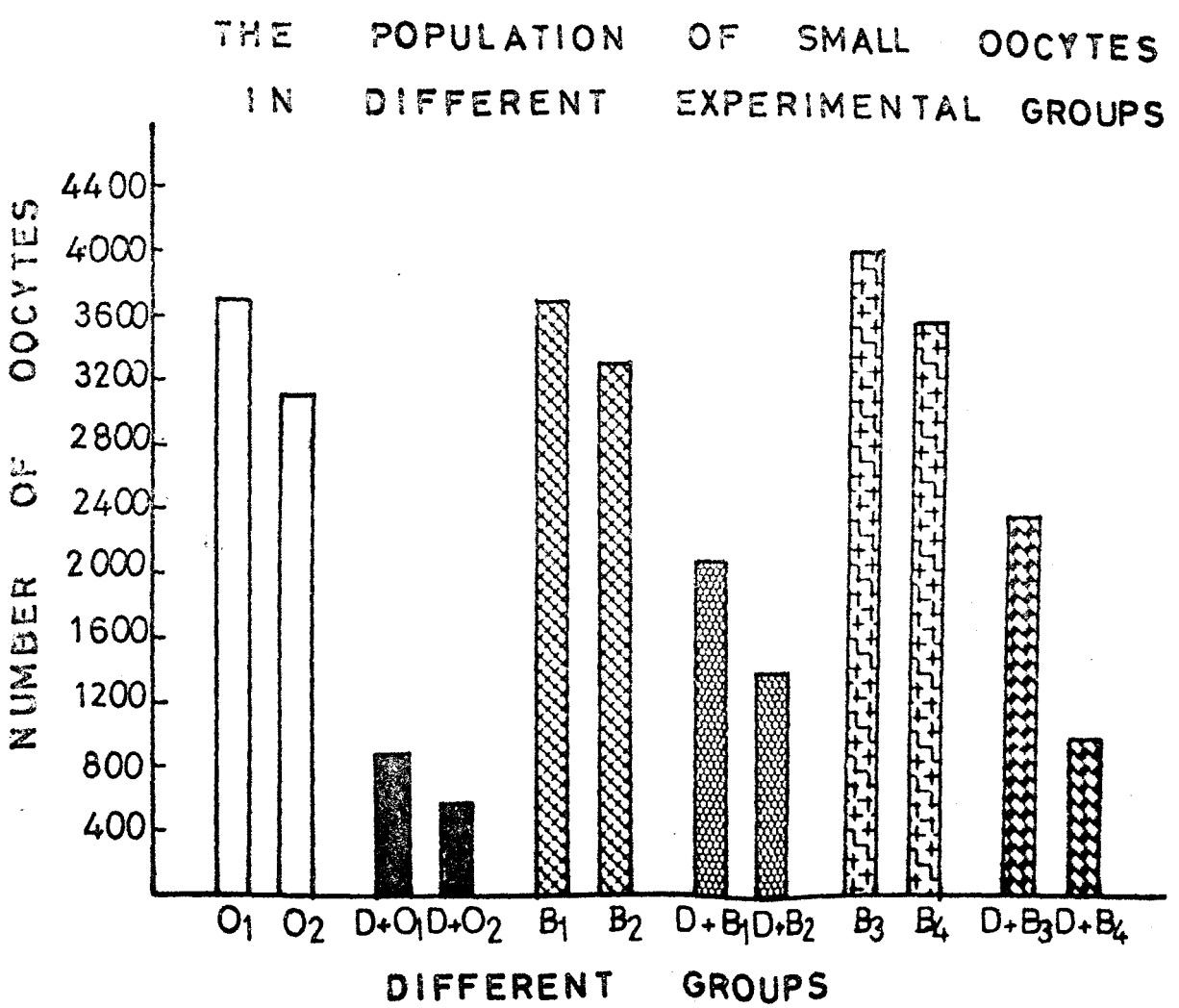

THE POPULATION OF SMALL OOCYTES
IN DIFFERENT EXPERIMENTAL GROUPS
NUMBER OF OOCYTES
4400
4000
3600
3200
2800
2400
2000
1600
1200
800
400
O1 O2 D+O1 D+O2 B1 B2 D+B1 D+B2 B3 B4 D+B3 D+B4
DIFFERENT GROUPS

FIGURE II

O_1	=	Control Group First Interval
O_2	=	Control Group Second Interval
$D+O_1$	=	DMBA Group First Interval
$D+O_2$	=	DMBA Group Second Interval
B_1	=	BHA 5 mg/g diet Group First Interval
B_2	=	BHA 5 mg/g diet Group Second Interval
$D+B_1$	=	DMBA + BHA 5 mg/g diet First Interval
$D+B_2$	=	DMBA + BHA 5 mg/g diet Second Interval
B_3	=	BHA 10 mg/g diet First Interval
B_4	=	BHA 10 mg/g diet Second Interval
$D+B_3$	=	DMBA + BHA 10 mg/g diet First Interval
$D+B_4$	=	DMBA + BHA 10 mg/g diet Second Interval

Fig : II

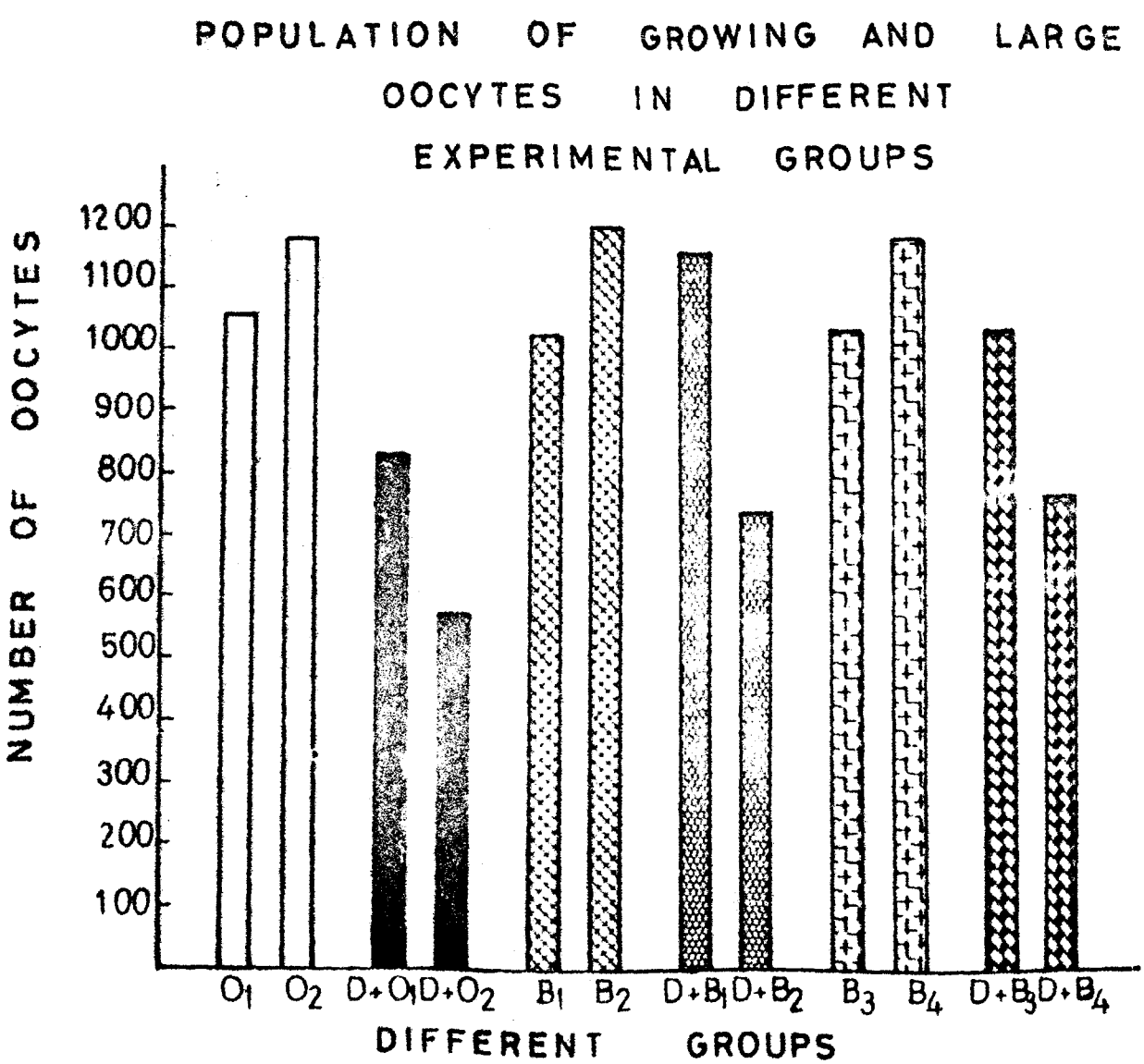

POPULATION OF GROWING AND LARGE OOCYTES IN DIFFERENT EXPERIMENTAL GROUPS
NUMBER OF OOCYTES
1200
1100
1000
900
800
700
600
500
400
300
200
100
O1 O2 D+O1 D+O2 B1 B2 D+B1 D+B2 B3 B4 D+B3 D+B4
DIFFERENT GROUPS

FIGURE III

O_1 = Control Group First Interval

O_2 = Control Group Second Interval

$D+O_1$ = DMBA group First Interval

$D+O_2$ = DMBA Group Second Interval

B_1 = BHA 5 mg/g diet Group First Interval

B_2 = BHA 5 mg/g diet Group Second Interval

$D+B_1$ = DMBA + BHA 5 mg/g diet First Interval

$D+B_2$ = DMBA + BHA 5 mg/g diet Second Interval

B_3 = BHA 10 mg/g diet First Interval

B_4 = BHA 10 mg/g diet Second Interval

$D+B_3$ = DMBA + BHA 10 mg/g diet First Interval

$D+B_4$ = DMBA + BHA 10 mg/g diet Second Interval

Fig : III

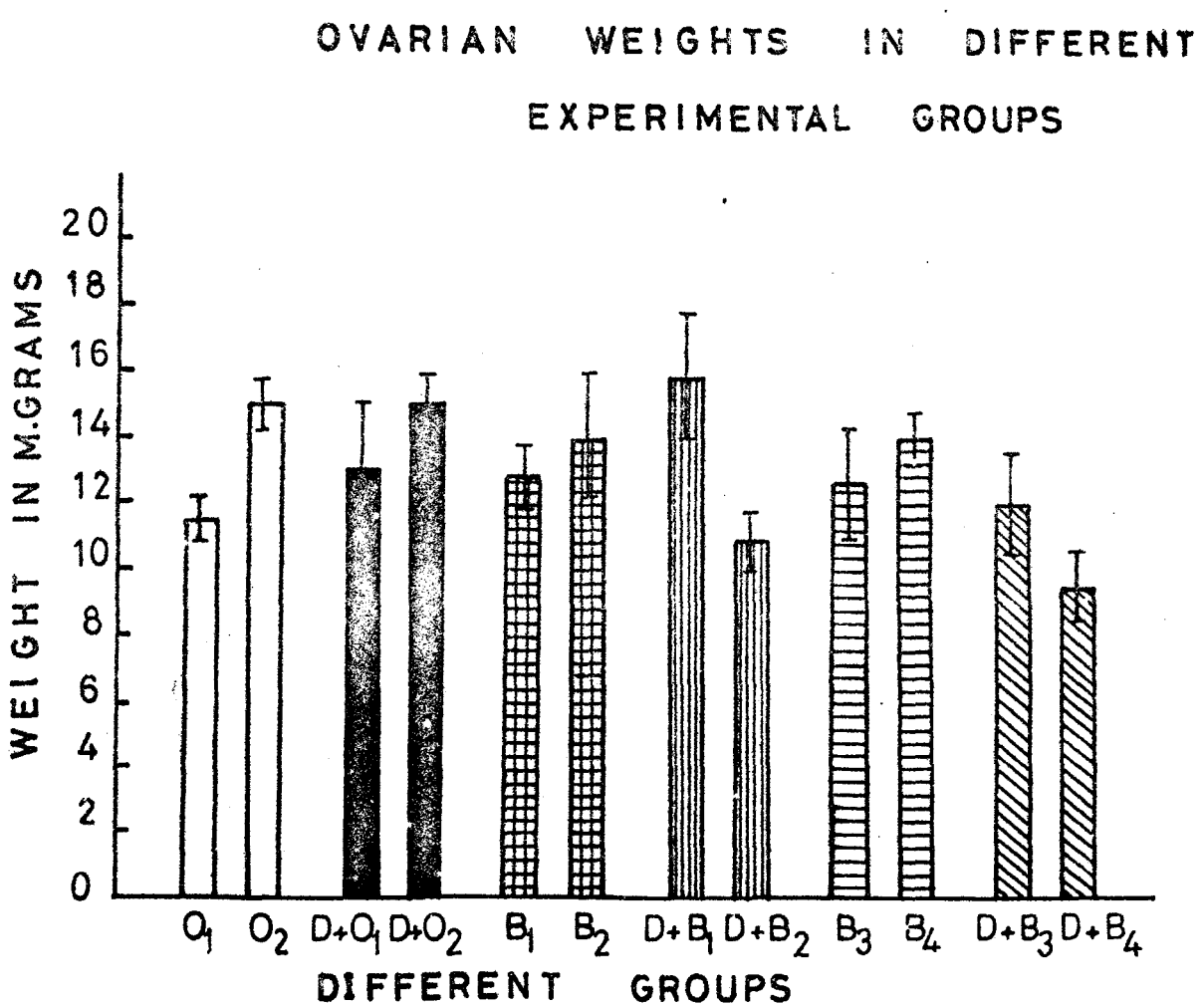
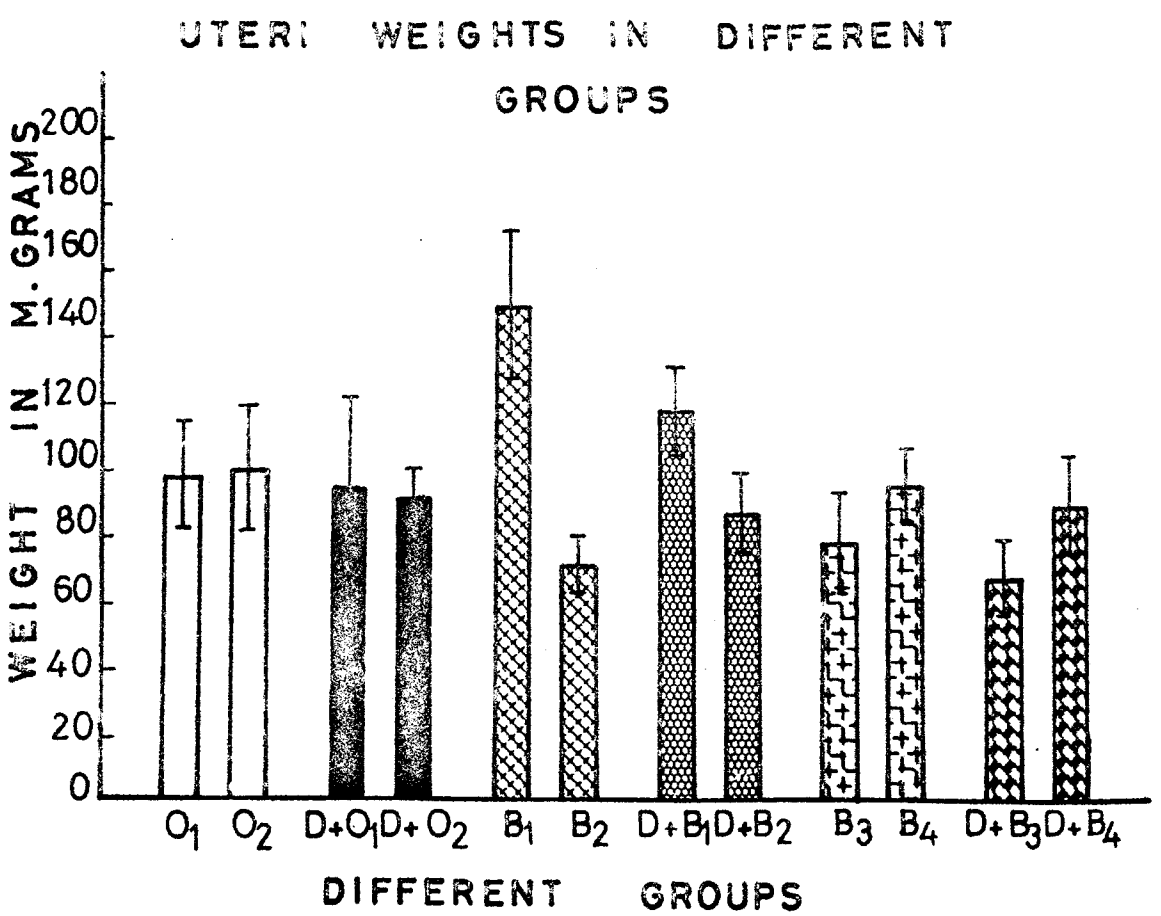

OVARIAN WEIGHTS IN DIFFERENT EXPERIMENTAL GROUPS
WEIGHT IN M.GRAMS
0
2
4
6
8
10
12
14
16
18
20
O1
O2
D+O1
D+O2
B1
B2
D+B1
D+B2
B3
B4
D+B3
D+B4
DIFFERENT GROUPS

FIGURE IV

O_1	=	Control Group First Interval
O_2	=	Control Group Second Interval
$D+O_1$	=	DMBA Group First Interval
$D+O_2$	=	DMBA Group Second Interval
B_1	=	BHA 5 mg/g diet Group First Interval
B_2	=	BHA 5 mg/g diet Group Second Interval
$D+B_1$	=	DMBA + BHA 5 mg/g diet First Interval
$D+B_2$	=	DMBA + BHA 5 mg/g diet Second Interval
B_3	=	BHA 10 mg/g diet First Interval
B_4	=	BHA 10 mg/g diet Second Interval
$D+B_3$	=	DMBA + BHA 10 mg/g diet First Interval
$D+B_4$	=	DMBA + BHA 10 mg/g diet Second Interval

Fig: IV.


UTERI WEIGHTS IN DIFFERENT GROUPS
WEIGHT IN M. GRAMS
200
180
160
140
120
100
80
60
40
20
0
O1 O2 D+O1 D+O2 B1 B2 D+B1 D+B2 B3 B4 D+B3 D+B4
DIFFERENT GROUPS

atretic follicles with a remnant of zonapellucida were also found.

Table III depicts the mean weights of ovaries and uteri at two intervals. Certain ovaries showed increase in their weight at 45 days interval which could be attributable to estresus cycle - associated changes in follicular growth. The uterus showed an insignificant increase of weight.

Group - II (DMBA)

This batch of animals exhibited remarkable changes in oocyte number as well as ovarian histology due to treatment. Table II represents marked depletion of SO (76%) at first interval. A further drop (35%) was monitored at the second interval. A lesser decline in the number of GLO at the first and second intervals as compared to that in the control, showed relative resistance of GLO to DMBA.

At 45 days the GLO count showed a decline (31%) in contrast to that in control group (Group I) where actually an increase (12%) was been.

In this group the percentage of GLO at 30 and 45 days intervals are about 48% and 50 respectively (Table Ia).

The ovarian histology during the first and second intervals showed remarkable degenerative and pathological changes in the follicles. Oocytes of all sizes were seen but spaces in the sub-epithelial layer apparently left by degenerated small oocytes were characteristic. Occasionally empty rings and psudofollicles could be observed in the outer cortex.

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

The mean weight of the ovaries was 13.12 mg at the first interval (Table III). It is an insignificant increase of 14% as compared with that at the first interval of control group. In the second interval the

ovarian weight increased but this increase was not significant. The uterine weight, on the other hand, showed some decline.

Group - III (BHA 5 mg/g diet)

It serves as control for group (DMBA + BHA - 5 mg/g diet). In this batch no significant depletion of SO and GLO populations were noticed at either interval. Similarly, the ovaries did not show any appreciable change in their weight. The uterine weight, showed appreciable increase during the first interval whereas during the second interval it showed noticeable decline (Table IV(a) and Figure IV).

Group - IV (DMBA + BHA 5 mg/g diet)

The SO were about 2080 in the first interval (Fig. I(a)) Thus the SO count significantly decreased when compared with that in BHA (5 mg/g diet) group (Group III). However, their population showed a significant decline in atresia when compared with DMBA group (Group II, Table I(a)

The difference in SO population between first and second intervals, this group is less than that seen between first and second intervals of the group, treated with DMBA alone (Table II(a))

On exposure to DMBA, SO population, as compared with that in control group (Group I) showed 76% and 82% decrease respectively at 30 and 45 days. It is interesting to note that when BHA is given along with DMBA, the depletion of oocytes is reduced very much. Thus, the oocyte population in Group IV declined only by 43% and 58% respectively at 30 and 45 days (see Group III for comparison).

The growing and large oocytes in the first and second intervals showed 40% and 29% increase respectively as compared with corresponding figures in Group II (TABLE II(b)). The mean weight of the ovaries were 15.94 and 10.87 mg at first and second intervals respectively.

The weight of the uteri was 119.49 at the first interval and it was reduced to 88.59 mg at the second interval. (TABLE III).

Group - V (BHA 10 mg/g diet)

In this group the number of SO did not show any appreciable alteration in their counts at either intervals (compare either with Group I or with Group III). In the first interval the small oocytes were 4020. And in the second interval the small oocytes were 3575. This decrease at the second interval was only 11% which falls in line with that in the control group I (i.e. 15) or Group III (i.e. 10%) (See Table II a & b)

The GLO count did not show difference as compared with that in Group I. In this group the growing and large oocytes were 1035 in the first interval and 1180 in the second interval. Similarly, at second interval also the GLO counts did not deviate much from that in control (Group I).

The mean weight of the ovaries of this group was 12.63 mg in the first interval. At second interval weight was 14.04 mg (Table III). The mean weight of the uteri was 78.70 mg in the first interval and in the second interval the uterus weight was 96.72 mg.

Group - VI (DMBA + BHA 10 mg/g diet)

This group showed a fall in the atresia of oocytes as in the case of Group IV. In the first interval, the SO (small oocytes) decreased by only 41% (as compared with that in Group V). TABLE IIb).

The DMBA induced depletion at 30th day interval was significantly ($P < 0.05$) inhibited by concomitant treatment of BHA (10 mg/g diet). Similarly, at 45 days interval also BHA offered appreciable ($P < 0.1$) protection against DMBA induced oocytic atresia. However, it has to be noted that BHA even at this dose level does not give full protection against DMBA insult in oocytes, (as indicated by significant differences in the oocyte counts between groups VI vs. V).

Also it is evident that higher dose of BHA in the diet gives better protection to the oocytes. The GLO count at the first interval is 1037, whereas at the second interval is 770.

DISCUSSION

Present series of experiments were designed to see the effect of polycyclic aromatic hydrocarbon (DMBA) on the ovary of mice and the modulatory role of BHA (a food additive) on this effect. It has been documented that olive oil which has been used as a vehicle for DMBA administration does not elicit any adverse effects on the ovaries (Manoharan and Rao, 1980). Already it has been shown by many others that olive oil treated animals do not differ in their oocytic number or ovarian histology from these of untreated animals (Krsup et al., 1969).

DMBA is a potent carcinogen and it elicits tumourigenesis in organs like mammary glands, ovaries, adrenal glands, kidneys, testes etc. This carcinogen needs biotransformation before it triggers neoplastic transformation in the target tissue or organ. The liver is known to take active part in this biotransformation process (Charles Heidelberger, 1975). However, other organs and tissues containing appropriate enzyme systems also bring about this kind of biotransformation. For example, the mouse ovary has been demonstrated to contain the microsomal monorygeneses and epoxide hydrases necessary

for the toxic and nontoxic pathways (Mattison, 1978; Oesch, 1977).

The atretic changes seen in the ovary of mice in the present experiments should have been brought about by DMBA or its metabolites formed in the host body. Not only topical application of DMBA on the ovary, but also intragastric intubation can elicit such changes (Kraup et al., 1969, 1970a; Shiba and Nishizuka, 1968; Jull and Russell, 1970; Kraup and Loff, 1971; Mattison, 1979; Manoharan and Rao, 1980; Rao, 1981).

In the present investigation DMBA given by intragastric intubation to the animals has affected the small oocytes of the ovaries and reduced their number. The oocyte destruction is very high at 30 days as well as 48 days after the treatment. This finding of the present study is in conformity with the observations of Kraup (1969) and Kraup and Loft (1971), Manoharan and Rao,(1980); Rao (1981).

With the process of germ cell elimination pathological changes develop concurrently in the ovaries. It has been suggested that the neoplastic development is secondary to the premature elimination of oocytes caused by the carcinogen itself (Kraup, 1967). 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; Murphy, 1972) and that, among the four strains of mice spontaneous ovarian tumours occurred only in that particular strain of mouse in which oocytic atresia was evident within the life span of the animals (Jones and Krohn, 1961).

The ovarian pathology showed many atretic granulosa cells in DMBA group when compared to that in the control group. Besides degeneration of oocytes the early post treatment changes include appearance of empty rings and pseudo follicles. These characteristic structures have been noted by several authors after X-irradiation and described as anovular follicles (Guthrie, 1958; Srivastava and Rao, 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 embryonic cells lying dormant in the ovarian stroma or (c) formation from the germinal (surface) epithelium (Thung et al., 1956).

Diffused luteinized tissue derived from confluent corporaluten 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, et al., 1954). The ovaries of DMBA group did not show any appreciable change in their weight.

When little older animals were exposed to DMBA ovarian weight loss was evident (Manoharan and Rao, 1980). Also long-term study indicates a significant reduction in the ovarian weight following a single exposure to DMBA (Rao, 1981). Changes in the weight of uteri did not show any meaningful trend.

Present study demonstrates that concomitant administration of BHA and DMBA to young adult mice would result in the decline in oocytic depletion. At 5 mg/gm diet dose level BHA inhibited oocyte-killing action of DMBA not very significantly ($P<0.1$) at 30 days interval and insignificantly at 45 days interval. Whereas at 10 mg/gm diet dose level, BHA elicited decline in oocyte-killing action of DMBA significantly ($P < 0.05$) at 30 days interval and not less significantly ($P<0.\emptyset1$) at 45 days interval. Thus, the protective action of BHA against DMBA-induced insult

in the oocytes was dependent upon its dose. Also it should be noted that the protective action of BHA is insignificant (at lower dose level) or less significant (at higher dose level) during the second interval of observation. This may be due to the expression of certain irrepairable damage (caused by the carcinogen in spite of the presence of BHA) which was not evident in our light microscopic examination of the oocytes (which, hence, appeared as "normal" while counting) at the early interval but succumbed to atresia by the second interval. Similar postulation has been put forward by Rao (1982) in a transplacental carcinogenesis study in mice.

There are many earlier reports of inhibitory effects of BHA on cancer incidences, particularly with situations where the route of administration resulted in direct contact of carcinogen with the target tissues like fore stomach, lung, skin, breast etc. (Wattanberg, 1972).

Some studies on the mechanism of inhibition of chemical carcinogenesis by BHA have been performed. Most of them involve the carcinogens like B(a)P or DMBA.

Generally polycyclic aromatic hydrocarbons are metabolised by the microsomal mixed function oxidase system which acts on a wide variety of xenobiotic compounds. Reactive metabolites as well as detoxification products are produced. The effects of administration of BHA on microsomal metabolism of B(a)P in female A/HCJ mice have been studied with experimental conditions similar to these in which BHA inhibits neoplasia due to this carcinogen.

Incubation of B(a)P and DNA with liver microsomes from the BHA fed mice results in approximately half the binding of B(a)P metabolites to DNA as compared to that found with microsomes from control mice (Speier and Wattenburg, 1975). Investigations were made by Wattenberg's group (Luk et al., 1977) to determine if the B(a)P metabolites that were employed when the carcinogen was incubated with liver microsomes prepared from BHA fed mice, differ from those formed in controls. It was found that the liver microsomes isolated from mice within four hours after administration of BHA showed a depression of B(a)P metabolism by more than 16%. A profound decrease

in the concentration of metabolites in the polar region of the chromatogram was noted indicating a radiation in the formation of diolepoxides by BHA. Also, it was found that the formation of B(a)P 4, 5-oxide was reduced with microsomes from BHA-fed mice (Wattenberg, 1977). The major metabolite in microsomal incubations from BHA fed and control mice was 3-HOBP. This metabolite constituted a significantly higher percentage of the total metabolites formed when B(a)P was incubated with microsomes from BHA fed mice as compared to the percentage in the control. Thus BHA alters microsomal metabolism by diminishing activation reactions leading to the formation of ultimate carcinogenic metabolites and also enhances formation of metabolites of detoxification.

Recently, it has been shown that treatment of animals with BHA decreased the amount of 7β, 8 -dihydroxy-9 x, 10 x - apoxy - 7, 8, 9, 10 - tetrahydrobenz(a)pyrene (BPDER): adduct in the lung and the liver approximately by 55 and 75% respectively (Anderson, Boroujerdi and Wilson, 1981).

SUMMARY AND CONCLUSION

1. The present investigation deals with the influence of BHA (a food additive) at two dose levels 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 BHA 5 mg/g diet Group

IV DMBA + BHA 5 mg/g diet Group

V BHA 10 mg/g diet Group

VI DMBA + BHA 10 mg/g diet Group

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

3. When a single dose (3 mg/mouse) of DMBA was administered to the mouse, the oocytic population, counted at the intervals of 30 days and 45 days, showed significant reduction at either interval.

4. BHA when given alone either at 5 mg/g diet level or at 10 mg/g diet level did not elicit any depopulation of oocytes.

5. When BHA and DMBA were given concomitantly to the mouse the oocytic depletion was reduced; the reduction in the depletion of oocytic population depended upon the dose of the modulator (BHA) and also the post-treatment interval; higher dose of BHA was more effective than the lower dose in protecting against DMBA-induced insult in the oocytes; this protective effect of BHA (at either dose level) was comparatively less at later interval.

6. The gravimetric changes in the ovaries and uteri did not depict any meaningful trend during the observation period.

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