INDUCTION OF CERVICAL CARCINOGENESIS BY METHYLCOLANTHRENE IN MOUSE
INTRODUCTION

Cervical carcinogenesis has been induced in a number of animal species using various carcinogens and different modes of treatment and this aspect is discussed elaborately in Chapter I. A detailed sequential analysis of MCA-induced cervical carcinogenesis has been performed in our laboratory using 600 µg of MCA each mouse which shows the tumor incidence in 90% of the mice in 70-90 days time (Manoharan 1982). In the present study methylcholanthrene (MCA), a polycyclic aromatic hydrocarbon (PAH), at the dose level of 300 µg/animal has been used for the induction of cervical tumors.

Unlike all the previous studies conducted in this laboratory where a carcinogenic dose of 600 µg of MCA, which yielded 70-90% cervical tumor incidence, was used for chemopreventive studies, in the present study a carcinogenic dose of 300 µg of MCA, which yielded 30% cervical tumor incidence, was used for assessing the chemomodulatory (augmentatory or inhibitory) influence of different hormonal contraceptive formulations.

STRUCTURE OF MOUSE UTERINE CERVIX

Leppi (1964) has described the structure of mouse uterine cervix (Fig. 17). The uterus of mouse consists of
Fig. 17. Diagrammatic representation of internal structure of adult mouse uterine cervix.

(From: Leppi, Anat. Rec., 150:51:1964)
two uterine horns which join caudally and form uterine corpus or body which gets further narrowed to form the uterine cervix. The uterine cervix further joins the vagina which receives a little portion of the former. The mid-dorsal and midventral outer surfaces of the cervix are fused with the adjacent inner surface of the vagina to form dorsal and ventral frenula. The two lumina of the uterine horns continue inside the corpus where it remains separated by a midline septum. These lumina then join caudally and form the cervical canal. Microscopically the lumina of the uterine horns and upper one-half of the corpus is lined with simple columnar epithelium with simple branched and tubular uterine glands projecting down from the lumen while rest portion of the corpus, the cervical canal and the projected portion of the cervix into vagina are made up of stratified squamous epithelium. Stratification and cornification of squamous epithelium is largely influenced by the stages of estrous cycle. The stroma of the cervix is composed of thick, interlocking bundles of compactly arranged collagen fibres. The inner layer of circular smooth muscle fibres and the outer longitudinal layer of smooth muscle fibres constitute the myometrium of each uterine horn. The longitudinal layer of smooth muscle fibres continue caudally only into the ventral and dorsal portion of the walls of corpus. The medial portion of each circular layer is merged to blend
with a mid-sagittal sheet of longitudinally arranged smooth muscle fibres that occupies a central position in midline septum. The circular layers of the peripheral portion fuse to from a thick circular layer which is subdivided into an inner and outer layer. The inner layer constitutes the main muscle component of the cranial two thirds of the cervix while the outer one forms the muscle layer of vaginal wall. Cranial portion of the external os is composed of collagen fibres and it lacks smooth muscle fibres.

CERVICAL CARCINOGENESIS

Studies on the induction of cervical carcinogenesis reveal that like other cancers, this also involves the occurrence of different precancerous and cancerous stages. These stages include hyperplasia, dysplasia, carcinoma in situ and microinvasive carcinoma (Richart, 1973; Christopherson, 1977; Koss, 1978). Variation in the timings of these stages and latent period of the cancer development has been observed depending on the type of carcinogen and the mode of its application. Murphy's string method (1953) has been found to have relatively shorter induction period.

A number of polycyclic aromatic hydrocarbon compounds prior to their mutagenic and carcinogenic actions need to be activated by oxidative enzymes (Miller and Miller, 1974).
Studies on the carcinogenic ability and extent of DNA binding of MCA revealed that 9, 10-diol-7,8 epoxides might be responsible for its carcinogenic or mutagenic actions. (Vigny et al., 1977; Thakker et al., 1978; King et al., 1977; Wood et al., 1978). Figs. 18 and 19 give the structure of MCA and its active metabolite.

MATERIALS AND METHODS

Randombred, 8-9 weeks old virgin Swiss albino mice were supplied by Animal facility, JNU, New Delhi and were maintained in an air-conditioned animal room providing standard food pellets (Hindustan Lever Ltd., India) and tap water ad libitum.

Chemicals

Methylcholanthrene (MCA) was obtained from Sigma, USA, hematoxyline from Merck, Germany and Eosin from BDH, England. Yellow variety of bees wax was obtained from Mysore (India) and filtered four times in molten state (60°C) to remove dust particles.

Tumor Induction

Murphy's string method (Murphy, 1953; 1961) as described by Manoharan and Rao (1984) was followed for
Fig. 18. Chemical structure of 3-Methylcholanthrene

Fig. 19. Chemical structure of active metabolite of 3-Methylcholanthrene
(9,10-diol-7,8 epoxide form)
cervical tumor induction. This method comprises following steps.

**Preparation of MCA plus beeswax mixture and threads**

Cotton threads of suitable thickness were cut into pieces of appropriate length, washed in distilled water for 12 hours and then immersed in ethyl alcohol for about 1 hour. A knot was put at the end of every thread and then each thread was weighed. About 0.7 cm of each knotted end of the thread was impregnated with molten beeswax and then weighed again. By subtracting post impregnation weight of the thread with preimpregnation weight the amount of wax in each thread was calculated.

Now a known amount of methylcholanthrene was mixed in the known amount of molten beeswax kept exactly at the melting point (60°C) so that each impregnated thread should have $\approx 300$ ug of methylcholanthrene. The mixture was stirred in the molten state for about 70 hours and special care was taken to regulate the higher temperature limit of 60°C. The knotted end of each washed thread was immersed in the carcinogen-beeswax mixture and then dried.

**Intracervical thread-insertion**

Figure 20 shows the insertion of thread into the canal of uterine cervix. In this process the animals were
Fig. 20. Schematic drawing of thread insertion.
anesthetised by ether anesthesia and fixed on a wooden platform exposing the ventral side of the body. Now a small slit was made in the abdominal wall and both the uterine horns were exposed gently by using a blunt sewing needle. A small blunt thin needle carrying the thread was gently inserted through vaginal opening and guided into the cervical canal smoothly without any obstacle. The tip of the needle was taken out at the junction of the two uterine horns and thread was drawn into the vagina until the wax-coated portion reaches the cervical canal and the knot remains exactly at the place where vagina touches the external os. Then the free end of the impregnated thread was loosely tied around the uterine horn. This method assures the continuous exposure of whole uterine cervix with the carcinogen.

EXPERIMENTAL DESIGN

The animals were divided into different experimental and control groups (Table 3). Animals of groups 1, 2 and 3 were kept without treatment and sacrificed respectively after 30, 60 and 90 days. The animals of groups 4, 5, and 6 were inserted intracervically with wax-only-impregnated threads and sacrificed after 30, 60 and 90 days respectively. Likewise the animals of groups 7, 8 and 9 were
inserted intracervically with carcinogen impregnated thread and sacrificed respectively after 30, 60 and 90 days. The animals were weighed initially at fortnightly intervals and at the time of autopsy.

The animals were sacrificed according to the above mentioned schedules and their uterine cervixes were fixed in Bouin's fluid for histopathological assessment of precancerous and cancerous lesions. The animals with threads missing or displaced were discarded at the time of autopsy.

PROCEDURE FOR HISTOPATHOLOGICAL PREPARATION

1. Fixation, Dehydration, Infiltration and Block Preparation

The uterine cervix and vagina of each animal were dissected out, freed from connective tissue and fatty tissue and fixed in Bouin's fluid for 24-72 hours depending upon the volume of the tissue (Photographs 1 and 2). The tissues were then dehydrated by passing through a graded series of ethyl alcohol (50%, 70%, 90%, 100% I and II each for 1/2 hour), cleared in xylene (xylene I and II each of 1/2 hour). The cleared tissues were placed in the xylene plus wax mixture and then in the molten paraffin wax (58-60°C) for infiltration. After giving two changes of molten paraffin (1
hour in paraffin I and 1 hour in II) the tissues were embedded in fresh paraffin wax.

The embedded cervix was then sectioned, at a thickness of 5-6 μ, in a plane parallel to the long axis of the organ.

2. Staining Procedure

The sectioned slices of the uterine cervix were spread on albuminised glass slides and then dried at 35-40°C. Then the sections were deparaffinized employing xylene (two baths each of 4 minutes). After this the slides were passed through graded concentration of ethyl alcohol (100%, 95%, 70%, 50% and 30% each of two minutes) and then in running water for 3 minutes. The slides were then dipped in Harris' hematoxyline for 1 minute. After this the slides were kept in running water for washing for about 10 minutes and then in 50% ethyl alcohol for 2-3 minutes, 70% ethyl alcohol for 2 minutes and eosin, prepared in 70% ethyl alcohol for 2 minutes. Afterwards the slides were passed through graded concentrations of ethyl alcohol (70% 1 dip, 95% 3 dips, 100% I and 100% II each for 5 minutes, 100% alcohol + xylene (1:1) for 2 minutes, xylene I and II each for 3 minutes).

3. Mounting of Stained Sections

The slides carrying the sections were taken from xylene with forceps. A streak of mounting medium (DPX) was placed
lengthwise on to the slide and covered with glass cover slip slowly starting from one side of the slide. The mounting medium gradually spreads through the whole area of the section. The slides were dried at room temperature for 2-3 days.

**Histopathological Analysis**

The sections of the uterine cervix of each control and experimental animal were observed under microscope (Leitz Orthopan Microscope). In the present study we have chosen certain precancerous and cancerous lesions as parameters for assessing the effect. These are as follows:

**Hyperplasia**: Characterized by increase in the thickness of the basal layer due to the increased and orderly proliferative activity. Keratinization and flattening of the epithelial cells persist at the surface.

**Dysplasia**: This is a lesion in which part of the thickness of the epithelium is replaced by cells having varying degrees of atypia. The cells display nuclear enlargement and hyperchromatism. The abnormal epithelial cells are disorderly arranged. Three grades of dysplasia, mild, moderate and marked can be envisaged according to the degree of cellular atypia and epithelial architecture.
Mild Dysplasia: The lesion involves lower one third of the epithelium and shows varying degrees of hyperplasia and focal areas of epithelial dysplasia. Loss of cellular polarity and regular stratification are minimal. Nuclei are always enlarged and darkly stained.

Moderate Dysplasia: The atypia is seen spreading to the lower two thirds of the epithelial thickness. Hyperplasia, hyperkeratosis and dyskeratosis are present in a wide area of epithelium. The degree of epithelial abnormality is intermediate between mild and marked dysplasia.

Marked Dysplasia: The atypia is very pronounced and there is loss of cellular polarity and the crowded cells have large darkly stained nuclei. The abnormal cells tend to be present in the upper third as well as middle and lower thirds of the epithelium. The lesion presents the picture of intraepithelial carcinoma except that there is a variable amount of differentiation of the superficial layer of the epithelium.

SQUAMOUS CELL CARCINOMA

a) Carcinoma in situ or Intraepithelial carcinoma: It shows a marked proliferation of cells in the entire epithelium where nuclei are enlarged and
hyperchromatic. There is an increased number of mitotic figures and crowding of atypical cells. However, the basement membrane remains intact and there is no invasion of the underlying stroma. Loss of stratification and polarity are seen.

b) **Invasive Carcinoma:** All the characteristics present in the squamous cell carcinoma in situ are seen in the invasive carcinoma. In addition there is disruption of basement membrane with sheets or islands of malignant squamous cells invading the underlying stroma. The invasive squamous cell carcinoma of mouse are further classified as:

- **Differentiated:** Tumors with much keratinization and pearl formation.
- **Poorly differentiated:** Tumors with some keratinization.
- **Undifferentiated:** Tumours without any keratinization.

All these precancerous and cancerous lesions have been depicted through photograph in several previous theses from this laboratory. Photomicrographs of only important lesions are presented here in this thesis (Photomicrograph 3-8).
RESULTS

The findings of the present study is depicted in Table 3. The animals of groups 1, 2 and 3 which were kept without any treatment and sacrificed respectively after 30, 60 and 90 days did not show any tumor incidence. Hyperplastic condition was observed in one of the animals in group 3. Likewise the animals of groups 4, 5 and 6 inserted intracervically with beeswax only impregnated threads and sacrificed respectively after 30, 60 and 90 days, also did not display any cervical tumor. However 1 out of 16 in group 5 and 4 out of 25 in group 6 developed hyperplastic conditions. The animals of groups 7, 8 and 9 which were treated intracervically with MCA (300 μg) plus wax-impregnated thread and sacrificed respectively after 30, 60 and 90 days displayed tumor incidences respectively as 0.0%, 8% and 27%. The tumors were carcinoma in situ or, differentiated, poorly differentiated and undifferentiated invasive types.

Out of 20 animals in group 7, 15 developed hyperplasia and 4 showed mild dysplastic condition while in group 8 out of 24 animals, 8 developed hyperplasia, 8 mild, 4 moderate and 2 marked dysplasia. In group 9 out of 22 animals 4 cases of hyperplasia, 5 mild, 3 moderate and 4 marked dysplasia were found.
### TABLE 3: METHYLCHOLANTHRENE-INDUCED CARCINOGENESIS IN THE UTERINE CERVIX OF MOUSE

<table>
<thead>
<tr>
<th>TREATMENTS</th>
<th>P EFFECTIVE</th>
<th>BODY WEIGHT (g)</th>
<th>NO. OF MICE</th>
<th>NUMBER OF MICE WITH CERVICAL LESIONS</th>
<th>TUMOR INCIDENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>G (INTRACERVICAL)</td>
<td>E NO. OF</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>R ROUTE</td>
<td>R MICE</td>
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<td>O</td>
<td>I INITIAL</td>
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<td>S (Gr.) (DAYS)</td>
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<td></td>
</tr>
<tr>
<td>1. NIL</td>
<td>30</td>
<td>15</td>
<td>21</td>
<td>25</td>
<td>15</td>
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<tr>
<td>2. NIL</td>
<td>60</td>
<td>15</td>
<td>20</td>
<td>28</td>
<td>15</td>
</tr>
<tr>
<td>3. NIL</td>
<td>90</td>
<td>15</td>
<td>20</td>
<td>30</td>
<td>14</td>
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<tr>
<td>4. WAX THREAD</td>
<td>30</td>
<td>15</td>
<td>20</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>5. WAX THREAD</td>
<td>60</td>
<td>15</td>
<td>19</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>6. WAX THREAD</td>
<td>90</td>
<td>15</td>
<td>22</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>7. MCA+WAX THREAD</td>
<td>30</td>
<td>20</td>
<td>21</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>8. MCA+WAX THREAD</td>
<td>60</td>
<td>24</td>
<td>20</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>9. MCA+WAX THREAD</td>
<td>90</td>
<td>22</td>
<td>20</td>
<td>29</td>
<td>0</td>
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|                            |              |                 |             |                                       |                  |
|                            |              |                 |             | INCIDENCE (%)                         |                  |
|                            |              |                 |             | CIS Carcinoma insitu; DIFF. = Differentiated, POORLY DIFF. = Poorly Differentiated, UNDIFF. = Undifferentiated. |

Dysplasia: + = Mild; ++ = Moderate; +++ = Marked.

Carcinoma insitu:
- CIS = Carcinoma insitu
- DIFF. = Differentiated
- POORLY DIFF. = Poorly Differentiated
- UNDIFF. = Undifferentiated
DISCUSSION

Strain of mouse used in the present study does not show any spontaneous tumor incidence. Present investigation using murphy's string method (Murphy, 1953) as described by Manoharan and Rao (1984) displayed 0.0%, 8.0% and 27% tumor incidence respectively after 30, 60 and 90 days of exposure with ~300 ug of MCA per mouse. Tumors were mostly invasive carcinoma with only one animal showing carcinoma in situ. Different stages of dysplasia were also recorded after different time intervals.

Among various types of carcinogen and methods of their application used for cervical tumor induction, methylcholanthrene (MCA)-string method has been found to be most effective giving the highest cervical tumor incidence in a short-span of time. Scarpelli and Von Haam (1960) produced 80% cervical tumor incidence in about 77 to 230 days using MCA-strings. Manoharan (1982) reported a maximum of 90% cervical tumor incidence in about 70-90 days after MCA (~600 ug)-thread insertion in mice. This high incidence compared to the previous studies has been related to the increased dose of MCA (i.e.~600 ug/animal). Another study in our laboratory (Das, 1986) in which the MCA-threads (~600 ug/thread) were removed after different time
intervals have shown that the tumor yield depends on the exposure and post exposure observation period. The maximum tumor yield was observed with 30 days exposure and 3-1/2 months post exposure period than other groups of animals where exposure and post exposure period was less (Das, 1986).

Higher tumor yield because of the long exposure period was explained as (a) Placement of 3-MCA containing wax-thread for longer period could increase the probability of interaction of more number of target cells with the carcinogen leading to the initiation in more cells. (b) MCA could act as an initiator as well as a promoter. Alauddin and Zaman (1967) in their work also reported the higher tumor yield when the exposure period was more.

The present as well as previous investigations suggest the involvement of a sequential multistep process in the development of invasive cervical cancer in mouse. But because of the overlapping of different stages, a very clear model for different steps had not emerged. This includes hyperplasia, dysplasia, carcinoma in situ and finally invasive carcinoma.

Equivocal opinions have been recorded regarding the fate of dysplasia in the process of cervical carcinogenesis.
Lange (1960) reported that 70 percent of cervical dysplasia advanced to carcinoma in situ. We come across a substantial evidence from the human studies also, suggesting the ultimate development of dysplasia into malignant tumors (Galvin et al., 1955; Peckham and Greene, 1957; Koss et al., 1963; Michalkiewicz et al., 1963). Study by Das (1982) is in agreement with these findings suggesting the progression of dysplasia into carcinoma. Christopherson and Broghamer (1961) also indicated that once dysplasia was produced, it progress to invasive carcinoma. However VonHaam (1961) observed the disappearance of dysplastic lesions after the withdrawal of carcinogen stimulus.

Extremely less incidence of CIS, observed in the experimental cervical carcinogenesis, is in contrast to the human situation where it is very common and remains for a longer period of time in the patient's life. Peterson (1956) reported that CIS continued to remain for almost 1 to 20 years before it progressed to invasive cancer.

Invasive carcinoma observed in the present study displays different degrees of keratinization which forms the basis of their further classification into differentiated, poorly differentiated and undifferentiated carcinoma.
Intracervical insertion of wax-only impregnated threads did not elicit any tumor induction in the present study; however, emergence of hyperplasia and mild dysplasia in few animals could be due to the sustained irritation of the cervical epithelium.
Photograph 1

Showing the caudal portion of the reproductive tract: uterine horns, cervix and vagina.

Photograph 2

Showing caudal portion of the reproductive tract with cervical tumor.
Photomicrograph 3

Longitudinal section of the normal cervical region showing stratified squamous epithelium (x 100).

Photomicrograph 4

Section of the cervical epithelium showing hyperplastic condition with proliferation of basal cell layer (x 100).
Photomicrograph 5

Cervical squamous epithelium of mouse showing dysplastic condition. Disorderly arrangement of the cells, disturb cellular polarity and hyperchromatic nuclei are prominent (x 100).

Photomicrograph 6

Showing the carcinoma in situ condition of cervical epithelium. Loss of stratification and polarity are seen. No differentiation of the entire layer. Hyperchromasia is prominent. Basement membrane intact (x 100).
Photomicrograph 7

Showing highly differentiated invasive carcinoma of the cervical epithelium. Masses of cancerous cells invading into the stroma with maximum keratinization and pearl formation (x 100).

Photomicrograph 8

Showing the poorly differentiated invasive carcinoma of the cervical epithelium. Keratinization is less (x 100).