AN ASSESSMENT OF THE BIOLOGICAL CHARACTERISTICS OF THE NEOPLASTIC CELLS OF ETHMOID CARCINOMA IN CATTLE

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled "An Assessment of the Biological Characteristics of the Neoplastic Cells of Ethmoid Carcinoma in Cattle" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled **"An Assessment of the Biological Characteristics of the Neoplastic Cells of Ethmoid Carcinoma in Cattle"** is a record of research work done independently by **Sri. B. Gangadharan** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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We, the undersigned members of the Advisory Committee of **Sri. B. Gangadharan,** a candidate for the degree of Master of Veterinary Science in Pathology, agree that the thesis entitled **"An Assessment of the Biological Characteristics of the Neoplastic Cells of Ethmoid Carcinoma in Cattle"** may be submitted by Sri. B. Gangadharan in partial fulfilment of the requirement for the degree.

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CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

Introduction

INTRODUCTION

The carcinoma of the mucosa of the ethmoid in domestic animals is an important neoplastic condition. Incidence of this neoplasm was reported from Scandinavian countries in the beginning of this century, although it probably does not occur at present in that country. Subsequently the incidence of this tumour was reported from different parts of the world. Muthappa in 1930 recorded the incidence of this tumour in cattle from Madras State for the first time in India. Since then, prevalence of this tumour was reported from Kerala, Tamil Nadu, Andhra Pradesh, Karnataka and also from Uttar Pradesh indicating that this condition has spread to different parts of the country. In recent years there has been considerable increase in the incidence of this condition. Its high incidence, lack of any species barrier and the specific location of the origin of the tumour single out this as an unusual neoplasm and this evoked keen interest among scientists associated with investigation in the field of Veterinary Oncology.

Among cattle, cross-breds in the age group of six to nine years are mostly affected. As the incidence is mostly seen in high yielding cows at the second trimester of pregnancy, it has emerged as an economically important disease which cause both high morbidity and mortality. Since the first record of

the tumour in Kerala in 1960, the incidence of this tumour is on the increase and now it has established itself in an endemic form affecting various species of livestock like cattle, buffalo, pig, goat and wild animals in captivity like the deer.

During the last ten years investigations were undertaken on various aspects of this problem at the Centre of Excellence in Pathology. Symptomatology was described, diagnostic criteria were established and pathological features were sequentially chartered. The immunological background of the tumour bearing cattle was pictured and the technique for cytological diagnosis was perfected. The fine structure of the neoplastic cells was studied in detail. Chemotherapy with endoxan was found to be of value in the early stages of the tumour growth. The studies conducted so far suggested the involvement of a virus in the causation of this tumour and the positive role of aflatoxin in the carcinogenesis was also suggested.

Although studies were undertaken on various aspects of this tumour, the investigations to establish a tumour cell line was not successful. An understanding of the nature and behaviour of the neoplastic cell is very essential to understand the mechanism of cancer development and to chalk out an effective strategy to control the unmanageable proliferative character of the tumour cell. Cell biology study is now being

widely used as a tool to understand the nature of the neoplastic process in oncology. Development of a cell line will be a milestone in the study of biology of any cancer. Previous studies conducted in this regard were not very successful and pointed out the requirement of some unknown factors to support the growth of ethmoid carcinoma cells in tissue culture media. Hence, an effort was made with improved media for successful development of a cell line of this tumour. Cultivation of tumour cells in artificial media alone will not speak of its biological character. Various organelle in the cell shows alteration depending upon the biological behaviour of the cell. Therefore, a study of the various cellular organelle will help to determine the biological character of the cell. Mitochondria are important organelle which shows changes in neoplastic process. Histochemical techniques are available to demonstrate mitochondria in routinely processed paraffin embedded sections. This technique to demonstrate mitochondria was included in this study to evaluate changes in the mitochondria of the neoplastic cell.

Gamma glutamyl transpeptidase (GGT) is an enzyme which is involved in the drug detoxification and its presence in the tumour cell is suggested to indicate the involvement of a genotoxic chemical carcinogen in the initiation of the tumour. Aflatoxin has been shown to be a genotoxic carcinogen and it was

suggested that aflatoxin might be involved in the genesis of this particular tumour. Therefore, histochemical demonstration of GGT was also undertaken as a part of this study, which will serve as an indirect evidence for the involvement of a genotoxic carcinogen, in the causation of this tumour.

Diagnosis of a tumour is an important step in the approach to the cancer therapy. A recently developed silver colloid technique to demonstrate nucleolar organiser regions (AgNORs) are now widely employed as a diagnostic tool in various neoplasms. Number of AgNORs per nucleus is an indication of ploidy and proliferative character of the cell. The AgNOR technique has been effectively used to differentiate various types of human neoplasms. Therefore, in this study the usefulness of AgNOR technique as a diagnostic test was assessed.

Trace elements like copper and zinc were reported to show a lower level in cancer patients. This reduction was used as a biochemical marker for early cancer diagnosis. Literature on this aspect is scanty in animal cancer patients. Hence, evaluation of serum copper and zinc levels was undertaken as a part of this study to assess their usefulness in early diagnosis of the condition.

Incidence of this condition is on the increase. Animals maintained in organised livestock farms are affected more. The

epidemiological data indicated a genetic pre-disposition. Parentage of the sinus tumour affected animals examined in the study was screened, to ascertain the genetic predisposition, if any.

Review of Literature

REVIEW OF LITERATURE

2.1 Incidence and epidemiology

Ethmoid carcinoma in cattle and horses was reported from Sweden in the beginning of this century (Moussu, 1906; Stenstrom, 1909, 1915; Forsell, 1913; Bergman, 1914; Magnusson, 1916; and Stalfors, 1917). Horne and Stenersen (1916) reported the incidence of ethmoid carcinoma in cattle from Norway. Jackson (1936) reported the incidence of ethmoid carcinoma in cattle from South Africa. Borkelhammer (1949) described an adenocarcinoma of the nasal epithelium in Shetland sheep dog. Cohrs (1952, 1953) reported the incidence of transmissible adenocarcinoma and infectious adenopapilloma of the olfactory mucosa of sheep. Cotchin (1956) in his review of the neoplasms of domestic animals cited 160 reports of ethmoid cancer in cattle and 24 in horses in Sweden. Bedford (1959) reported the incidence of adenocarcinoma in the maxillary nasal sinus in the dog. Young et al. (1961) reported various neoplasms arising from the olfactory mucosal membrane of the sheep. Amaral and Nesti (1963) observed the carcinoma in the ethmoidal sinus of cattle and pigs in Brazil. Nazario et al. (1966) reported carcinoma in the ethmoidal sinus of bovines and swines. Rubaj and Wolosyn (1967) reported enzootic adenopapilloma of the nasal cavity in sheep. Duncan et al. (1967) reported enzootic nasal

adenocarcinoma in sheep. Tokarnia et al. (1972) reported enzootic ethmoid tumour in cattle in Rio-de-Janero, State of Brazil. Becker et al. (1972) observed nasal tumours in cattle. Inada et al. (1973) and Inada and Tokarnia (1973) described ethmoid tumours in pigs and cattle respectively. Bradley and Harvey (1973) made a brief note on the incidence of nasal tumours in dogs. Cho et al. (1974) reported adenocarcinoma in the nasal cavity and brain of a dog. Legendre et al. (1975) reported the incidence of nasal tumours in cats. Brownstein et al. (1975) reported nasal carcinoma in a captive eld's deer (Cervus eldi). Mason (1975) recorded a case of spindle cell sarcoma of the equine paranasal sinus and nasal chamber. Stanzi and Hauser (1976) described tumours of the nasal cavity in domestic animals. Madewell et al. (1976) observed 300 cases of primary neoplasm involving the nasal cavity or paranasal sinuses of domestic animals among 12,300 cases studied. The multi species data were compiled from abstracts of medical records maintained by 13 colleges of veterinary medicine in the United States and Canada from 1964-1973. Brown et al. (1977) reported a case of nasal adenocarcinoma in a Taiwan Macaque. Confer and Depaoli (1978) observed sixteen cases of primary neoplasm of the nasal cavity, paranasal sinuses and nasopharynx in the dog. Yonemichi et al. (1978) reported intranasal tumour of the ethmoid olfactory mucosa in sheep. Njoku et al. (1978) reported the incidence of ovine nasal adenopapilloma from ten

sheep. Pospichil et al. (1979, 1982) reported five cases of endemic ethmoid carcinoma in cattle and studied their histological and untrastructural character. Howard et al. (1982) mentioned about the descriptive epidemiology of the carcinoma of the nasal cavity and paranasal sinuses in the dog. Stroud and Amurdson (1983) reported a case of squamous cell carcinoma perforating the hard palate and reaching the nasal cavity in a free ranging white tailed deer (Odocoileus virginianus). Njoku and Chineme (1983) reported the neoplasms of the nasal cavity of cattle and sheep. Giauffret et al. (1984) reported nasal tumours in caprines. Adant et al. (1984) reported a case of congenital ethmoid carcinoma in a foal. Steen et al. (1985) reported nasal tumours in a fallow deer (Dama dama L). Ringe and Rajko (1985) observed growth in the nasal cavity as naturally occurring nasal obstructions in sheep. Charray et al. (1985) described an outbreak of adenocarcinoma of the olfactory mucosa in West African Dwarf ewes. The report by Haltgren et al. (1987) described the features of fibro-sarcoma in the nasal and maxillary sinuses of two young horses, of which one was congenital, and a case of spindle cell sarcoma in a young horse. Wendt (1989) described incidence, clinical aspects and diagnosis of ethmoidal tumour in sheep. He studied 11 cases during a period of 10 years. Morrison et al. (1989) reviewed the case records at Mardoch University Veterinary Hospitals over the period of 1978-1988, and it revealed 37 cases of nasal tumour in dogs.

2.1.1 Incidence and epidemiology in India

In India Muthappa (1930) was the first to record a case of neoplasm in the ethmoid mucosa of a cow. David and Venkataraman (1940) reported the occurrence of malignant growth in the frontal sinus of cattle. Nair and Sastry (1954) in a survey of 2003 neoplasms in domestic animals in Madras State recorded 18 cases of ethmoid cancer in cattle and buffalo. Narayana (1960) reported a case of Carcino Sarcoma of nasal passage and frontal sinus in an eight year old Ongole breeding bull from the state of Andra Pradesh. Sastry and Rao (1964) recorded a case of tumour consisting of adenocarcinoma with a fibro-sarcomatous stroma in a bullock. Rajan et al. (1972) reported the incidence and pathology of tumours of paranasal sinuses in domestic animals in Kerala. Damodaran et al. (1974) reported fifty one neoplasms in the ethmoid region of bovines from the state of Tamil Nadu. Balasubrahmaniyam (1975) studied seventeen cases of sinus tumour in bovine from the state of Karnataka. Prasad and Kohli (1978) recorded a case of nasal osteoma in a bullock. Nayak et al. (1979, 1980) reported tumours of bovine nasal cavity from Orissa. Jayaraman et al. (1979) stated that in the recent years there has been an increase in the incidence of neoplasm involving ethmoid region in domestic animals. They did not find any breed specificity but neoplasms were common in the age group of 6-9 years. There was preponderence of incidence in the progeny of few sires

indicating genetic predisposition. Epidemiological features of the disease indicated an infectious etiology. The tumours of the mucosa of the ethmoid were encountered in Kerala since 1960. One hundred and fifty cases were recorded during 1977-1979 period. It consisted of cattle (133), buffalo (7) and goats (10) (Rajan, 1980 and Rajan and Sulochana, 1982). A survey undertaken in Tamil Nadu during a period from 1977-80 revealed the occurrence of the disease more in organised farms where livestock were kept congregated (Viraraghavan et al. (1980). Rajan et al. (1980, 1981) reported ethmoid carcinoma in goats and pigs. Choudhary and Rao (1982) recorded 60 cases of tumours of the ethmoid mucosa and paranasal sinuses from cattle and buffalo during a period of 8 years from 1974 to 1981. Pruthi et al. (1982) recorded a case of fibrosarcoma of nasal region in a bullock. Sreekumaran and Rajan (1983) observed that this tumour has established itself in an endemic form in Kerala. They recorded high incidence in cross-bred Jersey cattle particularly in the age group of 7-10 years. All districts in the state were equally affected. Rameshmurthi (1984) reported the incidence of ethmoid carcinoma in Jersey cattle in the Red dane project in Karnataka. The animals were in the age group of 4-11 years. Kornel et al. (1984) reported the incidence in a herd of purebred Jersey in the age group of 5-11 years. Bovine leukemia virus antibody was detected in 10 out of 21 cases but its relationship to ethmoid neoplasm was not clear. The

possibility of a vertical transmission of the neoplasm in the herd was discussed. Singh and Singh (1984) recorded a case of adenocarcinoma in the nasal cavity of a buffalo. Mahdi (1985) reported a case of squamous cell carcinoma in the nasal cavity of a mare. Murali Manohar et al. (1986) described a case of ethmoid carcinoma in a goat. Swarup et al. (1987) reported two cases of ethmoid carcinoma in adult dairy cows from Uttar Pradesh. Chakrabarthi et al. (1988) described three cases of ethmoid carcinoma in HF cattle from West Bengal. Murali Manohar (1988) made a survey of occurrence of ethmoid neoplasm in domestic animals for a period of 5 years (1983-1987) covering cattle, buffalo, sheep, goat and dog.

2.2 Diagnostic procedures

2.2.1 Clinical symptoms

Persistent nasal discharge either bilateral or unilateral with or without blood, dyspnoea and unilateral or bilateral exophthalmos were the common clinical symptoms (Moussu, 1906; Stenstrom, 1915; Muthappa, 1930; David and Venkatraman, 1940; Narayana, 1960; Tokarnia et al. 1972 and Jose et al. 1985). Snoring and abdominal type of respiration were found in advanced cases (Rajan et al. 1972; Nair, 1973; Damodaran et al. 1974; Balasubrahmaniyam, 1975; Njoku et al. 1978; Jayaraman et al. 1979). Nervous symptoms like circling movement, cachexia,

perforation of the frontal bone and bulging of the forehead were reported by Nayak et al. 1979; Pospichil et al. 1979; Sreekumaran, 1980; Rajan et al. 1981 and Pruthi et al. 1982. Swelling of the sub-maxillary lymphnodes was reported by Kornel et al. (1984). Murali Manohar (1988) reported nasal discharge, epistaxis, exophthalmos, frontal swelling, dyspnoea, and nervous symptoms as the clinical symptoms of this condition.

2.2.2 Exfoliative cytology

Nair (1973) observed neoplastic cells in the nasal discharge. The neoplastic cells had hyperchromatic nucleus with clumping of chromatin, anisokaryocytosis and anisocytosis. Cells in mitotic division were also observed. Masillamony et al. (1978) advocated staining of nasal smears using acridine orange as a tool for diagnosis. Later the results of study conducted by Masillamony et al. (1980) employing acridine orange, indirect flourescent microscopy and papinicolaou stain revealed that it could be used as an effective tools of exfoliative cytology and diagnosis for picking up cases of sinus neoplasms at an early stage. All the three methods gave positive correlation with post mortem findings. Vijayan (1981) screened nasal discharge from 105 cattle, 15 goats and 13 pigs for identifying cases of ethmoid carcinoma. papinicolaou stain, Shorr's stain, methyl green pyronin stain, haemotoxylin and eosin and Wright's stain were employed to study the

cytomorphology of the cell. Papinicolaou stain was found to be the method of choice for a precise diagnosis of carcinoma. Haematoxylin and eosin and Wright's stain were found to be satisfactory for preliminary screening. The cytological diagnosis and typing of the tumour by exfoliative cytology were confirmed by histopathological examination and the usefulness of exfoliative cytology in the diagnosis of ethmoid carcinoma was established. Vijayan and Rajan (1982) perfected the diagnostic criteria employing papinicolaou stain for exfoliative cytology. Murali Manohar (1988) established the role of exfoliative cytology using Acridine Orange and Papinicolaou stain in the early diagnosis of the tumour.

2.2.3 Other methods

Jayaraman et al. (1980) employed various serological tests for the diagnosis of sinus neoplasm in bovines. Cytotoxic tests and virus neutralisation test yielded encouraging results. Gel diffusion test and hypersensitivity reactions did not yield satisfactory results. Rajan and Vijayan (1981) evaluated the usefulness of Ehrlich's test as a diagnostic test. The results were encouraging and they suggested the usefulness of this test as one of the battery of tests to be employed for diagnosing the tumour. Sulochana and Rajan (1981) tried Agar Gel diffusion and passive haemaglutination tests for the diagnosis of endemic ethmoid carcinoma in cattle. They evaluated this test using

materials from 56 tumour animals and were found to be not useful in the diagnosis. Vijayan and Rajan (1982b) prepared mucous block from cattle bearing ethmoid carcinoma by collecting mucous using a nasal scoup and by aspiration. Aspiration method gave satisfactory results. An organoid pattern of the tumour tissue was evident and a precise diagnosis of the tumour was possible. Vijayan and Rajan (1982c) evaluated the cerebrospinal fluid of the animal bearing ethmoid carcinoma and recorded slight lymphocytic pleocytosis. Murali Manohar (1988) stated that radiology and ultra sound technique also were useful in the diagnosis. Murali Manohar and Sunderaraj (1989) employed mucous block technique to detect ethmoid carcinoma in 14 cases. Thirteen cases showed positive results correlating with the post-mortem finding and suggested that this technique can be effectively employed as a diagnostic tool to detect early cases of ethmoid neoplasm in cattle. Koshy et al. (1989) also suggested the usefulness of radiological techniques in the diagnosis and monitoring of the treatment using chemotherapeutic agents like cyclophosphamide. Murali Manohar et al. (1990) suggested the use of sialic acid and lipid associated sialic acid in the serum as a reliable biochemical marker for tumour detection.

2.3 Histopathology

2.3.1 Cattle

Adenocarcinoma, squamous cell carcinoma and undifferentiated carcinoma were the common types observed (Stenstrom,

1915; Nair and Sastry, 1954; Rajan et al. 1972; Nair, 1973; Damodaran et al. 1974; Balasubrahmaniyam, 1975; Pospischil et al. 1979; Jayaraman et al. 1979; Sreekumaran 1980; Rajan, 1980; Rajan and Sulochana, 1982; Choudhary and Rao, 1982; Sreekumaran and Rajan, 1983; Chakrabarti et al. 1988 and Murali Manohar, 1988). Other types of tumours encountered were osteoma (Moussu, 1906), myxosarcoma (Moussu, 1906 and Nayak et al. 1979), transitional cell carcinoma (Nair, 1973 and Balasubrahmaniyam, 1975), fibroma (Muthappa, 1930), mixed cell sarcoma (David and Venkatraman, 1940), carcinosarcoma (Narayana, 1960 and Sastry and Rao, 1964), fibromyxo-chondro osteoma, myxochondro osteosarcoma and fibro-osteo chondroma (Becker et al. 1972) histiocytic tumour, malignant lymphoma and reticulam cell sarcoma (Balasubrahmaniyam, 1975), fibrosarcoma (Madewell et al. 1976; and Choudhary and Rao, 1982), osteoma (Prasad and Kohli, 1978), atypical osteoma (Rumbaugh et al. 1978) and mesenchymal blastoma (Pospischil et al. 1982).

2.3.2 Other species

Young et al. (1961) and Duncan et al. (1967), reported intranasal tumours of epithelial origin in sheep. Cho et al. (1974) reported adenocarcinoma in the nasal cavity of a dog. Adenocarcinoma in the captive Eld's deer was reported by Brownstein et al. (1975). Mason (1975) described a spindle cell sarcoma in the equine paranasal sinus and nasal chamber.

Brown et al. (1977) described a papillary adenocarcinoma in a Taiwan macaque monkey. Yonemichi et al. (1978) grouped the intra nasal tumours of sheep as papillary adenoma or adenocarcinoma. Njoku et al. (1978) also reported papillary growths of epithelial cells in the nasal cavity arising from the mucosa of the ethmoid bone in sheep. Confer and Depaoli (1978) reported respiratory epithelial carcinoma, adenocarcinoma, squamous cell carcinoma, mucoepidermoid carcinoma, undifferentiated carcinoma, chondrosarcoma, and undifferentiated sarcoma in dogs. Rajan et al. (1980) observed adenocarcinoma, papillary adenocarcinoma, and squamous cell carcinoma in goats. Adenocarcinoma was the histological type of ethmoid carcinoma observed in pigs (Rajan et al. 1981). Stroud and Amurdson (1983) reported squamous cell carcinoma in a free ranging white tailed deer, in the nasal cavity. Adand et al. (1984) observed a congenital ethmoid anaplastic carcinoma in a foal. Mahdi (1985) reported a squamous cell carcinoma in the nasal cavity of a mare. Wendt (1989) reported papillary adenoma, fibroadenoma and adenocarcinoma from the nasal cavity of sheep.

2.4 Nucleolar organiser regions (AgNORs)

Nucleolar organiser regions are chromosomal segments in which ribosomal RNA (r RNA) is encoded and they are thus responsible for the development of RNA containing nucleolus or nucleoli into which the NORs project on large loops of DNA.

Such AgNORs in the mammalian chromosomes were visualised using a Silver Staining Technique (Good Pasture and Bloom, 1975). Busch et al. (1979) observed large numbers and unusual arrays of nucleolar argyrophilic granules in Novikoff hepatoma, KB and Hela cells, with the aid of a simple silver staining procedure. The nature of argyrophilic elements in the nucleolus was not known to them. Howell et al. (1980) readily demonstrated these regions in metaphase chromosome spreads by means of an argyrophil technique. Buys and Osinga (1980) observed abundance of protein bound sulphhydryl and disulfide groups of chromosomal AgNORs, by selective silver staining. The relative abundance of r RNA genes facilitated their detection in chromosome spreads or intact nuclei by r RNA/r DNA in situ hybridization. In human karyotype NORs were located in each of the short arms of the acrocentric chromosomes, 13, 14, 15, 21 and 22 (Stahl, 1982). Ploton et al. (1982) localised ultra structurally the NORs in nucleoli of human breast cancer tissues using a one step AgNOR staining method. Silver staining does not identify neither r RNA nor r DNA, but the acidic proteins associated with these sites of r RNA transcription. These proteins are designated B_{23}, C_{23}, AgNOR protein and RNA polymerase I (Ploton et al. 1986). They improved the staining and visualisation of argyrophilic proteins of the AgNORs at optical level and observed that NOR numbers appeared to reflect cell and nuclear activity. According to Fakan et al. (1986) abundance and

intensity of AgNORs were an indication of not only their absolute number and dispersion but also their transcriptional activity. Crocker and Nar (1987) observed that there was significant difference between the number of AgNORs in low grade lymphoma and high grade lymphomas. Low grade lymphoma had 1-1.5 AgNOR/nucleus and high grade lymphoma had 4.4 to 6.8/nucleus. Crocker et al. (1987) made a quantitative study on the AgNOR associated proteins in cutaneous melanotic lesions. Egan et al. (1987) studied AgNORs in the small cell tumours of childhood. They observed AgNOR numbers of 12.96 for neuroblastoma, 6.7 for round cell rhabdomyosarcoma, and 9.66 for ewings sarcoma. On the basis of the number of AgNORs one could differentiate between the above three types of tumours. The difference observed were statistically significant. The subtypes of rhabdomyosarcoma could not be distinguished from one another on the basis of AgNOR counts. They suggested that this simple histo-chemical technique may be a useful tool to the range of special technique now available to assist the pathologist in the diagnosis of small round cell tumours. Crocker et al. (1987) studied the AgNORs in small cell carcinoma of the bronchus. Egan and Crocker (1988) applied this silver staining technique to 68 cutaneous tumours including basal cell carcinoma, eccrine tumours, apocrine tumours, dermatofibromas and squamous cell carcinomas. Among this, basal cell carcinoma, eccrine tumours, apocrine tumours and hair follicle tumours had

difference in their number of AgNORs; these appeared as small black dots in their nuclei. Dermatofibromas and squamous cell carcinoma showed a degree of variability in the number of AgNOR's depending on the cellularity of the former and differentiation of the later. Basal cell carcinomas possessed significantly many more AgNOR per nucleus than the other neoplasms. It was suggested that this technique, previously the providence of cytogeneticist, may be of use in the diagnosis of cutaneous neoplasms. Giri et al. (1988) studied AgNOR's in benign and malignant epithelial lesions in the breast. They observed the following number of AgNORs in various lesions, epitheliosis 2.21 ± 0.31; papilloma 1.92 ± 0.24; sclerosing adenoma 1.96 ± 0.21; intraduct carcinoma 3.75 ± 1.33; lobular carcinoma in situ 2.67 ± 0.54; invasive carcinoma 4.22 ± 1.42. Twenty one of the 28 intraduct and invasive carcinoma with an AgNOR count of 4 or greater contained an aneuploid population but 15 of the 29 tumours with an AgNOR count of less than 4 also revealed aneuploidy. There was a trend towards higher AgNOR counts with increased proliferation indices. They suggested that AgNOR counts were of limited value in distinguishing epitheliosis from intraduct carcinoma. However, in malignant lesions, AgNOR counts may provide a prognostic index independent of tumour ploidy. Fallowfield and Cook (1988) observed a highly statistically significant difference between the AgNOR numbers in benign naevus cells (1.18 ± 0.05) and atypical melanocytes of

melanocytic dysplasia (1.17 ± 0.08) and in malignant melanocytes (7.64 ± 0.47). A single large AgNOR was seen in most naevus cells and atypical melanocytes of melanocytic dysplasia, whereas multiple small AgNORs were seen scattered throughout the nuclei of malignant melanocytes. This difference in nuclear staining was so striking that precise counting was regarded as unnecessary. They suggested that this technique should be of value in the diagnosis of borderline melanocytic lesions. Denham and Solisbury (1988) studied AgNORs in keratoacanthoma and squamous cell carcinoma. They observed AgNOR count of 2.0 in the range of 1.4 to 2.6 in keratoacanthoma and 2.1 in the range of 1.4 to 3.2 in squamous cell carcinoma. They opined that, there seems to be no relationship between NOR counts and clinical growth rate in keratoacanthoma and squamous cell carcinoma. The AgNOR count may be yet another reflection of the similiarities between the tumours. McNicol et al. (1988) studied AgNORs in pitutary adenomas. They noted 1.45 ± 0.07 AgNOR in normal pitutary, 2.18 ± 0.09 in macro adenoma, 1.69 ± 0.11 in microadenoma, 1.73 ± 0.14 in prolactinoma and 2.18 ± 0.15 in corticotroph adenomas. Crocker et al. (1988) stated that there was a very good linear correlation between the mean numbers of AgNOR sites per nucleus and percentage of S phase cells in non Hodgkins lymphoma. DNA flow cytometric values and AgNOR counts were high in high grade Non-Hodgkins lymphoma and low in low grade lesions. Conversely there was no significant

correlation between the DNA index, representing DNA aneuploidy and AgNOR counts. They suggested that the number of AgNOR in a lymphoma may be related to dividing fraction of a cell rather than as might be expected to ploidy alone. Crocker et al. (1988) evaluated the AgNOR technique in the diagnosis of malignant mesotheliomas. Normal mesothelial cell showed an AgNOR count of 1.04 ± 0.11, Reactive cell had 1.75 ± 0.55, Tubulo papillary type mesothelioma had 5.43 ± 1.34, Undifferentiated mesothelioma 5.00 ± 1.23, Sarcomatous mesothelioma 7.52 ± 2.56 and mixed type of mesothelioma had 4.94 ± 0.125. Crocker et al. (1988) made a comparative study of AgNORs, Ki 67 staining and DNA flow cytometry in Non-Hodgkins lymphoma. They confirmed that AgNOR numbers were greater in high grade than in low grade Non-Hodgkins lymphomas. It was shown that there was a high linear correlation between the number of tumour cell reacting with Ki 67 and mean number of AgNOR per nucleus. They suggested that AgNOR method may atleast be used as an adjunct to Ki 67 and flow cytometry in the characterisation of lymphomas. Ohri et al. (1988) observed that the AgNOR dots was significantly higher in malignant breast lesions compared to that of benign lesions. The physiological significance of changes in the number of silver stained AgNORs in the interphase nuclei is unclear. Hormonal stimulation in vitro was shown to increase the mean AgNOR count in the primary culture of sensitive cells, but in vitro effects of increased hormonal activity have not

been studied. Following bilateral adrenolectomy (Ady) in the rat, the excessive hypothalmic stimulation resulted in an increase in the pitutary adrenocorticotrophin content as a result of cell proliferation and augmented activity of individual corticotrophs. There was significant difference between the number of AgNOR after adrenolectomy. The difference between the counts at 2 and 6 weeks after adrenolectomy was also significant. These increases are more likely to be related to changes in peptide synthesis than to cell proliferation, since the maximal expansion of the corticotroph population occurs before two weeks after adrenolectomy (Peebles et al. 1988). Kinsey et al. (1988) studied AgNOR counts in mucinous ovarian tumours. They recorded mean AgNOR numbers as 1.4 in benign group, 4.4 in borderline group and 5.4 in malignant group. The results of their experiment showed, that AgNOR counts generally correlated with malignant potential but exhibited too much overlap between groups to be useful in predicting prognosis in an individual case. Boon and Sharif (1988) evaluated the prognostic value of the AgNOR technique in Meningioma. They recorded 2.9 $\pm$ 0.84 AgNOR count in recurrent meningioma, 2.3 $\pm$ 0.30 in typical meningioma and 3.26 $\pm$ 0.69 in atypical meningioma and opined that this technique appears to be useless for predicting the biological behaviour of meningiomas. Williams et al. (1988) studied the DNA content and AgNORs in ocular melanoma. Average number of NOR per cell ranged from

2.08 - 7.4 in 49 tumours. Out of this 40 tumours were found to be diploid; 7 were aneuploid, whereas 5 were hyperdiploid by DNA content. Cromie et al. (1988) proposed a modification for the silver staining procedure. Coumbe et al. (1988) studied AgNORs in endometrial hyperplasia and neoplasia in human beings. Cystic hyperplasia showed 0.83 - 1.23 number of AgNOR, architectural atypia showed 0.96 - 1.35, cellular atypia showed 2.19 - 2.69 and adenocarcinoma had 2.12 - 2.37. The scores of glandular hyperplasia with cellular atypia fell within the same range as adenocarcinoma.

Smith et al. (1988) studied the effect of series of fixatives on the AgNOR technique. They showed that, in general alcohol based fixatives gave optimal results, Carnoy's fluid being especially recommended. Mercurial and dichromate containing fixatives were found to have highly detrimental effects on NOR staining. Routine 10% formal saline fixatives gave adequate results whereas 10 per cent neutral buffered formalin gave optimum staining similiar to alcohol based fixatives.

Underwood and Giri (1988) suggested that the higher AgNOR counts reported in certain tumours represented not so much an absolute increase in the numbers of AgNORs, but are attributable rather to increased transcriptional activity and nucleolar dispersion both of which make it easier to identify

and count individual AgNORs. Smith and Crocker (1988) evaluated AgNORs in breast malignancy in human beings. Crocker and Egan (1988) studied the correlation between NOR sizes and numbers in Non-Hodgkins lymphoma. In low grade specimens, the AgNOR sites were highly significantly larger than in high grade Non-Hodgkins lymphoma. Howat et al. (1988) employed two methods of counting silver stained black dots in nuclei. They concluded that this technique was of no value in predicting prognosis for cutaneous malignant melanoma. Leong and Raymond (1988) demonstrated AgNOR related proteins in microwave fixed tissues and compared the intensity of AgNOR staining technique in formalin fixed tissues. Boldy et al. (1989) applied the AgNOR staining method to cell imprints from lymphoid tissue and compared with that seen in 3 μ paraffin sections. The mean AgNOR count per nucleus was higher for imprint preparations. Giri et al. (1989) examined 214 benign and malignant breast lesions. They concluded that AgNOR method alone did not offer a reliable histological discriminant for malignancy in the breast. However, AgNOR counting provided information on breast cancer prognosis supplementary to that obtained from DNA flow cytometry. Rosa et al., (1989) evaluated the role of AgNOR to discriminate between reactive and preneoplastic lesions in gastric epithelium. Crocker et al. (1989) perfected the technique of counting AgNOR dots and stressed to pay attention to the rigorous technique and careful resolution of intra-nuclear AgNOR dots. They suggested that timing of reaction and

fixation methods were also important. Ruschoff et al. (1989) also applied AgNOR technique to cell imprints and they supported the view of others that, results on cell imprints were superior to those obtained on sections. Jan-Mohammed et al. (1989) concluded that the interphase NOR number is related to factors other than chromosome numbers, and suggested that NOR numbers at interphase may be related to the cell turn over. Murray et al. (1989) designed a technique which enabled the sequential demonstration of NORs, and various antigens in both frozen and paraffin embedded sections. The NORs was demonstrated by the standard argyrophil technique and the antigens were shown by either immunoperoxidase or immunoalkaline phosphatase methodology. They obtained clear reproducible results. It was suggested that this sequential method may be of great use in the evaluation of AgNOR numbers in neoplasms, where cell population was heterogeneous. Cell population may be demarcated with accuracy prior to the counting of AgNORs. Griffith et al. (1989) investigated the value of one stage silver staining technique for AgNORs as a predictor of clinical outcome in 100 rectal adenocarcinomas. They concluded that, assessment of NOR activity was not reliable in routinely formalin fixed archival tissues. Tidsley et al. (1990) studied 94 cases of colorectal carcinoma. There was no significant correlation between AgNORs and tumour grade or stage, nor did they correlate with the prognosis. Leopardi et al. (1990) evaluated the use of AgNOR technique in distinguishing benign from malignant mesothelial

cells in pleural and pericardial fluids. They showed no statistically significant difference between each other. An experiment conducted by Napier et al. (1990) explained the lack of correlation between quantitative expression of AgNORs, and the histological appearance and regression in an adriamycin treated experimental animal tumour. Suresh et al. (1990) observed that the AgNOR counts in non-neoplastic trophoblastic tissue were clearly a reflection of ploidy rather than of cell proliferation. Rosa et al. (1990) showed that AgNOR scores were higher in aneuploid tumours, but the difference was not significant. Rowlands et al. (1990a) described an alternate technique for staining AgNOR associated protein employing polyethylene glycol as the protective colloidal developer, instead of gelatin. This technique was found to be quick and easy to perform. Rowlands et al. (1990b) also evaluated the use of different fixatives like acetone, absolute alcohol, methanol, Carnoy's fluid, Bouin's fixative, 4 per cent gluteruldehyde, neutral buffered formalin and formal saline. AgNORs were easily demonstratable in frozen sections than in paraffin sections. Alcoholic fixatives were found superior to aldehyde fixatives, though the difference between the fixatives were not great. Ofner et al. (1990) stated that the evaluation of silver stained particles according to their different distribution pattern was of great value with regard to the clinical outcome of colonic carcinoma. Mean number of scattered AgNORs per nucleus and

staging of the tumour were strongly related. Ruschoff et al. (1990a) summarised their experiences with the silver staining of AgNORs in a total of 580 tumours from ten different tissues. They made use of automatic image analysis for the evaluation of AgNORs. This provided good reproducibility as determined by the standard cumulative means. The most suitable silver reaction time which rendered a good diagnostic difference in the AgNOR content of benign and malignant tissue ranged from 23 to 35 minutes in the breast cancer specimens. In another study by Ruschoff et al. (1990b) revealed the importance of staining time and their relation to diagnostic and prognostic significance. Staining of AgNOR was controlled by internal staining standards and the silver incubation time was decreased until the AgNORs were visible as distinct dots within the nucleoli. Leek et al. (1990) in a study assessed the relationship of AgNOR size and number to proliferative status in a range of renewing and neoplastic tissue, and concluded that expression of AgNOR was clearly dependent on proliferative status of the tissues, but not in an identical manner.

2.5 Tissue culture studies

Bernad (1878-1879) formulated the theoretical principle for the creation of an artificial system in which organs could survive outside the influence of the whole organisms. Roux (1885) made the first successful in vitro transplant. He kept

alive a chick medullary plate in physiological saline. Ljunggren (1897-98) kept fragments of human skin alive for several days and weeks in ascitic fluid. Beebe and Ewing (1906) grew the explants of dog lymphosarcoma in the serum of immunised or non-immunised animals. Harrison (1907) aseptically removed small fragments from the wall of the frog neural crest and cultivated them in coagulated frog lymph. In this system active growth continued for several weeks, and he observed growth of nerve fibre from the central body toward the periphery of the explant. Volpino (1910) cultivated cells from mouse adenocarcinoma in artificial media and found that these cells reproduced tumour when reinoculated into the mouse. Carrel and Burrows(1911) grew in vitro chicken Rous sarcomata, which also were transplanted into homologus species. Lewis and Lewis (1911) introduced the first liquid medium with some controlled components, such as different salts and peptones. They also used sea water, serum and aqueous embryo extract in different concentrations. In this media, the cells grew as a thin layer, excellent for microscopic observation, but only for a short period. Carrel (1912) introduced surgical methodology in the handling of tissues, particularly aseptic and antiseptic technique. He studied composition of media by identifying different substances favouring cell growth. He perfected the instrumentation for tissue culture work. Vogelaar and Erlichman (1933) successfully introduced the first synthetic media composed of peptone, hemin, cystine, insulin, thyroxine and

glucose. They used trypsin to detach cells from the walls of roller tubes. Morgan et al. (1950) perfected the synthetic tissue culture media and the synthetic media now-a-days used had their origin from their contribution. Moscana (1952) digested a fragment of chicken embryo in a 3 per cent solution of trypsin and observed that the cellular clumps obtained were still able to grow in vitro. Dmochowski et al. (1958) and Sykes et al. (1959a, 1959b, 1959c and 1961) grew cells from bovine ocular squamous cell carcinoma of different histological type. They included benign precancerous lesions, papilloma and carcinoma. Kasza (1964) established and characterised canine thyroid adenocarcinoma and canine melanoma cell lines. Duncan et al. (1967) reported failure to propogate the cells in vitro from neoplasm of the nasal mucosa of the sheep. Cleaver et al. (1972) cultivated cells from bovine ocular squamous cell carcinoma. Oughton and Owen (1974) established cell lines from dog neoplasms and transplanted into nude mouse. Owen et al. (1977) made tissue culture and transplantation studies on canine mammary carcinoma. Jun et al. (1978) reported success rate of 71 per cent in their attempt to establish cell line from ovine squamous cell carcinoma. They made no attempt to culture tumour near vulva and only explant technique was employed. Hoffman (1978) initiated cultures from bovine ocular squamous cell carcinoma. The cultures developed cytoplasmic vacoulation, but viruses were not demonstrated by electron microscopy. Yonemichi et al. (1978) demonstrated viral particles, which were

morphologically similar to the visna-maedi virus, in tumour tissues and in 3-4 cultures of intranasal tumours of ethmoid olfactory mucosa in sheep. Kleinschuster et al. (1979) cultured epithelial cells from bovine ocular carcinoma and observed structures similar to epithelial pearls seen in carcinoma. The effort to cultivate cells of bovine ethmoid cancer was not successful (Pospischil et al. 1979; Jayaraman et al. 1979 and Sulochana,. 1980). Owen et al. (1981) reported cell lines developed from various neoplasms of domestic animals. Semple et al. (1982) developed multiple cell lines from patients with malignant melanoma, and studied their morphology, karyology and biochemical parameters. Else et al. (1982) studied the characteristics of canine mammary carcinoma cell line. Rasheed (1983) characterised a cat melanoma cell line. Heeney et al. (1983) studied the ultra structural characteristics of bovine ocular squamous cell carcinoma grown in artificial medium. Al-Yamen and Willenborg (1984) successfully isolated and cultured naturally occurring ovine squamous cell carcinoma cells in tissue culture medium. Norval et al. (1984) studied the in vitro properties of three canine mammary carcinoma cell lines. Karki and Rajan (1986) tried to culture ethmoid carcinoma cells obtained from cattle and goats in HBSS, TC 199 (Difco) and Dulbecco's modified Eagle's medium (Difco) with 10-20 per cent foetal calf serum supplementation. The tumour cells obtained from cows failed to grow in all the media used.

The tumour cells from goats grew and could be maintained for 9 days. Subsequently the cells died even after subculturing and changing of media. The possible role of endogenous infective agent, degenerated condition of the tumour tissue and absence of certain unknown factors required by the neoplastic cells for growth were attributed as factors responsible for failure. Wolfe et al. (1986) achieved 43 per cent success rate in obtaining cell lines from canine mammary carcinomas and studied their biological character. Wolfe et al. (1987) established eight canine melanoma cell lines from tissues of six dogs with spontaneous primary or metastatic melanomas. Cell lines were characterised for morphologic features and growth patterns on plastic, pigmentation, ultrastructure, cloning efficiency in soft and tumourigenicity in nude mice. Inove et al. (1988) established a chicken monocytic leukemia cell line. Welling et al. (1988) developed a continuous cell line from a dog with leukemia. The cells were considered T lymphocytic in origin, because they did not produce immunoglobin, did not phagocytize, but did agglutinate with concanavalin A, PHA and pock weed mitogen. Strandstrom et al. (1989) established culture of canine mandibular salivary glandular epithelium and passaged more than 250 times. Eva Hellmen (1989) characterised four in vitro established canine mammary carcinoma and one atypical benign mixed tumour cell lines. The cell line developed so far had been cultured for about two years and passaged between 45

and 200 times. Retrovirus associated particles were found in two carcinoma cell lines. Cheney et al. (1990) derived a lymphoma cell line from a feline large granular lymphoma obtained from the abdominal mass of a 13 year old castrated male cat. The cells resembled natural killer precursor cells and had membrane bound granules.

2.6 Gamma Glutamyl Transpeptidase activity

Gamma glutamyl transpeptidase (GGT) hydrolyzes γ-glutamyl amides and catalyses the transfer of the γ-glutamyl group from γ-glutamyl peptides to other peptides, L-amino acids and water (Hanes et al. 1950, 1952). Goldbarg et al. (1960) demonstrated presence of GGT in normal human serum employing calorimetric method and a synthetic substrate. They also reported characteristic changes in the serum enzyme in liver disease. Albert et al. (1961) and Glenner et al. (1962) demonstrated GGT in tissue sections using histochemical procedures. Aronsen et al. (1965) observed high GGT activity in liver tissue adjascent to the tumour growth. High values were encountered in obstruction contrast to the lesser elevation found in acute hepatocellular disorder. Kokot et al. (1965) studied GGT activity in tumour diseases and suggested that liver tumour itself contributed to the serum GGT levels and they found uniform distribution of serum GGT activity in all globulin fractions. Rutenburg et al. (1969) evolved a new technique with γ-glutamyl, 4-methoxy

2-naphthylamide (GMNA) as the substrate for histochemical and ultrastructural demonstration of GGT. Aronsen et al. (1970) evaluated the value of GGT as a screen test for liver tumour and found that, in patients with hepatic secondaries, that of other enzymes commonly elevated in cholestatic hepatic disorders and used in the detection of hepatic secondaries. GGT was induced in rat liver following oral phenobarbitone or ethanol administration (Ideo et al. 1971), although, they did not find elevated plasma levels in short term studies. Ideo et al. (1972) also reported increased level of serum GGT in rats treated with carbon tetrachloride (CCl_4) at the dose rate of 0.5 ml/kg. Fiala et al. (1972) reported that in rats, neoplastic and preneoplastic liver cells seemed to contain increased amounts of GGT than normal hepatocytes during chemical carcinogenesis. Fiala and Fiala (1973) showed GGT activity in rat and mouse liver activated by Ethionine, a chemical carcinogen. Delarue et al. (1973) found GGT elevation to be invariable in cancer patients with either clinical or scintigraphic evidence of liver secondaries. It was not clear from their data whether elevation in such subjects truly reflected hepatic secondaries and normal values reflected their absence. Kalengayi et al. (1975) observed that expression of GGT was a common finding in liver lesions induced by Aflatoxin B_1, a genotoxic carcinogen. Rosalki (1975) reviewed the use of GGT in the diagnosis of disease and stated that GGT was accepted as an enzyme of importance, in the diagnosis of liver diseases,

and proved to be the most sensitive of the enzyme in a variety of liver disease. Fiala et al. (1976) observed an increased GGT activity in liver within 24-48 hours after a single dose of carcinogen, Dimethyl Nitrosamine (DMN). Cameron et al. (1978) and Pugh and Goldfarb (1978) reported increase of GGT in lesions of liver induced by a chemical carcinogen, 2- acetyl amino flourene (2-AAF), and they observed that this elevation served as a useful marker for evaluation of progression of the carcinogenic process. Deyoung et al. (1978) demonstrated GGT in normal and neoplastic mouse skin and explained their significance. Solt and Shklar (1982) observed rapid induction of GGT rich intraepithelial clones in 7, 12 Dimethyl benzanthracine treated hamster buccal pouch. In domestic animals, GGT activity was mainly seen in the kidney, pancreas and intestine. Its activity with liver was relatively high in cows, horses, sheep, and goats and very low in dogs, cats and birds (Braun et al. 1983). They also suggested that plasma reference values can help to interpret the variations of serum GGT mainly in hepatobiliary diseases of cattle, sheep, goats and cholestatic disorders of dogs. Urinary GGT was a good test of kidney toxic damage. Leonard et al. (1984) designed a study to investigate the specificity of serum GGT activity with the use of cell type specific hepatotoxicants in rats. CCl_4, Allyl alcohol and alpha naphthyl isothiocynate were used to produce cell-specific injury in centrilobular hepatocytes, periportal

hepatocytes, and bile duct cells respectively. CCl_4 and allyl alcohol administration had no effect on serum GGT activity. Administration of ANIT in the diet at 0.01%, 0.022%, 0.047% and 0.1% for 2, 4 and 6 weeks produced dose and time dependent increase in serum GGT activity. This increase correlated well with bile duct cell damage. Calderon (1985) demonstrated GGT activity in precancerous lesions and carcinoma of oral, pharyngeal and laryngeal mucosa in human beings. Chen et al. (1985) identified GGT activity not only in esophageal neoplastic foci but also in regions of both dysplastic and basal cells near tumour foci. Greaves et al. (1986) showed large increase in GGT activity in hepatic nodules induced by Diethyl nitrosamine (DEN). Chen et al. (1987) found GGT positive cells in 95 per cent of abrasive balloon exfoliative cytology collections from 113 patients with esophageal cancer. GGT positive normal esophageal squamous epithelial cells were frequently present along with squamous cancer cells. Braun et al. (1987) reviewed the uses of GGT in experimental toxicology and stated that increase in serum GGT was induced by various drugs and chemicals. Chen et al. (1988) demonstrated GGT activity in exfoliated cells from baloon collections from esophagus in the area with high incidence of esophageal cancer. Beer et al. (1988) demonstrated GGT $_M$ RNAs in liver tumour cells and preneoplastic cells in rats fed with Diethylnitrosamine (DEN) followed by phenobarbital. Rao et al. (1988) demonstrated lack of expression of GGT $_M$ RNAs in liver tumours induced by

peroxisome proliferator, non-genotoxic carcinogens like ciprofibrate and nafenopine. Yeldandi et al. (1989) indicated that administration of 2-Acetyl aminoflorene had no influence on the expression of GGT in liver lesions induced by ciprofibrate. Chen et al. (1989) evaluated the localisation of GGT in exfoliative esophageal specimens in a population of 911 individuals aged 35 or older who lived in an area of China with a high incidence of esophageal squamous cell carcinoma. Out of the total population 24.4 per cent was positive for GGT in which 10.4 per cent normal, 22.0 per cent hyperplastic, 42.4 per cent type I dysplastic 46.0 per cent type II dysplastic, 100 per cent near carcinoma and 77 per cent squamous cell carcinoma showed GGT positive reaction. They suggested that the presence of GGT positive cells in normal esophageal squamous epithelium proved useful for identification of dysplastic cells and this served as a diagnostic marker for patients who progressed to the carcinoma stage.

2.7 Mitochondria

Bensely and Hoerr (1934) in their study of cell structure showed that 30 per cent of the wet weight of isolated mitochondria was phospholipids. Copper phthalocyanine had a specific affinity for phospholipids with strong choline base (Pearse, 1955). Baker (1958) stated that phospholipids in the mitochondria were stained in paraffin sections by different dyes like altman's Acid fuchsin, Benda's crystal violet, Baker's acid

haematin and Sudan Black. Shanklin and Nassar (1959) employed luxol fast blue MBS as a 0.1 per cent solution in 0.05 per cent acetic acid for staining mitochondria of rat kidney. PAS was the counter stain used. Fleischer et al. (1961) and Getz et al. (1962) observed that 40 per cent of the structural material of the mitochondria was lipid. This consisted of 90 per cent phospholipids, especially lecithin and cephalin, although variations were found among different organs. Salthouse (1962) attributed the staining action of luxol fast blue MBS to the high solubility of this dye in certain phospholipids, such as lecithin and cephalin. Engel and Cunningham (1963) developed an improved trichrome technique for screening muscle biopsies for mitochondrial abnormalities. But it not only stained mitochondria red, but also stained nuclei, sarcoplasm, reticular myelin sheaths and several inclusions. The applicability of luxol fast blue MBS as a mitochondrial stain was confirmed in various organs of mouse and bull frog (Takaya, 1967). The mitochondria were stained green and differentiation with 5 per cent phosphotungstic acid was employed. Sordahl et al. (1969) and Pederson et al. (1970) stated that mitochondria in tumours were defective functionally. Nair (1980) studied the ultra structure of ethmoid carcinoma cells. He found that the size, shape and number of mitochondria varied in the tumour cell. Generally the mitochondria were swollen with the matrix having a granular appearance. Transverse and ring shaped cristae were noticed in some mitochondria, while in others there was complete

disorganisation and dissolution of the cristae. In some cases there was a preponderance of mitochondria, in which the strands of endoplasmic reticulam were arranged concentrically around the mitochondria. Horikoshi et al. (1987) employed a flourescent dye, Janus Green B to monitor the mitochondrial changes associated with malignant cell transformation. Nair et al. (1987) in a detailed ultra structural study of ethmoid carcinoma cells found varied number of mitochondria. They were round or oval or slightly elongated with prominent cristae or ring shaped. Transverse or ring shaped cristae were noticed in some mitochondria, while in others, there was complete disorganisation and dissolution. Another feature in some cells was ballooned cristae. In these cases there was no separation between inner and outer mitochondrial membrane. Expanded material chambers and disruption of the mitochondrial membranes were also seen. Although, in general mitochondria were randomly distributed, occasional close association between rough endoplasmic reticulam and mitochondria was seen. There was instances of curved endoplasmic reticulum, partially or completely encircling mitochondria with a narrow zone of cytoplasm persisting between them. Demartins et al. (1987) detected mitochondrial clusters in mammalian white fat cells using fluorescent dyes like Rodamine 6 h and 123. Tanaka et al. (1987) raised antibodies directed against isolated mitochondrial antigens, respiratory chain complex I and IV, and demonstrated this antigens in frozen sections using

immunohistochemical procedures. Paulus et al. (1990) reported results obtained using a monoclonal antibody M-1168, which recognised inner mitochondrial membrane in routinely processed paraffin embedded tissue by light microscopic immunocytochemistry.

2.8 Serum copper and zinc level

An alteration in the serum zinc and copper levels in human sarcoma patients were reported by Fisher et al. (1973) and suggested that determination of serum copper level and serum zinc level in osteosarcoma patients may be of value in prognosis and therapy evaluation. The importance of trace elements in cancer was reported by Schwartz (1975). Abdulla et al. (1978) reported a decrease in serum zinc and copper values in the sera of patients with head and neck tumours. Vyas et al. (1982) reported an elevation in the serum zinc and copper levels in malignancy. Varghese et al. (1987) reported a significant reduction in the serum copper and zinc levels in both human oral submucous fibrosis and oral cancer. The copper/zinc ratio was found to be elevated in oral submucous fibrosis and depressed in oral cancer. This ratio was pointed out to serve as a good indicator for the early detection of oral cancer.

2.9 Genetic predisposition to ethmoid carcinoma

Jayaraman et al. (1979) stated that in the recent years there has been an increase in the neoplasm involving ethmoid

region in domestic animals. They did not find any breed specificity, but neoplasms were common in the age group of 6-9 years. There was preponderance of incidence in the progeny of few sires indicating genetic predisposition. Kornel et al. (1984) reported the incidence of ethmoid neoplasms in a purebred Jersey herd in Orissa. They discussed the possible chance of vertical transmission of this tumour, as 80-100 per cent of the progenies of two bulls in the farm developed ethmoid carcinoma, and even their grand-daughters died due to this neoplasm.

Materials and Methods

MATERIALS AND METHODS

3.1 Screening of nasal discharge

Nasal smears of suspected animals sent by Veterinary Officers of the Department of Animal Husbandry, Government of Kerala and Officers in charge of the University Farms were stained with Wright's stain as described by Koshy et al. (1989) and examined under the oil immersion objective of the microscope. The positive animals were transported from the owners premises to the Centre of Excellence in Pathology and maintained.

3.2 Collection of tumour tissue

The tumour bearing animals were euthanised by exsanguination after stunning with a captive bolt pistol. The head was bisected into two halves with an electric saw. Healthy tumour tissue devoid of necrotic areas was dissected out from the deeper portions under aseptic precautions. Head lymphnodes like retropharyngeal, parotid and mandibular were examined for metastatic growth, if any. Detailed post-mortem examination was conducted to find out other lesions in the animal. For tissue culture studies fresh tumour tissue was collected in Hank's Balanced Salt Solution supplemented with high dose of antibiotics as follows:

Penicillin 600 IU/ml

Streptomycin 300 µg/ml

Gentamycin 150 µg/ml

Nystatin 300 IU/ml

For histochemical demonstration of AgNOR and mitochondria, tumour tissue was collected in 10 per cent buffered neutral formaldehyde and for demonstration of GGT, tumour tissue was collected in acetone and stored at 4°C.

3.3 Tissue culture studies

Fresh tumour tissue collected for tissue culture studies was washed twice in HBSS containing antibiotics and divided into two parts. One part was employed for explantation culture and the other part for dissociated cell culture.

3.3.1 Collection of foetal calf serum and goat serum

Male bovine calf born at the University Livestock Farm, Mannuthy and deprived of colostrum and goats slaughtered at the Municipal slaughter house, Trichur were used for collection of blood. Blood was collected under aseptic precautions from these animals without anticoagulant, and blood was allowed to clot. Clotted blood was kept in the refrigerator for maximum clot retraction and serum yield. The serum thus collected was heat

inactivated for 30 minutes at 56°C and filtered through Seitz Filter. The filtered serum was stored under refrigeration with aseptic precautions at ⁻10°C.

3.3.2 Sterilization of glasswares

All the glasswares and stainless steel equipments like Seitz filter and scissors were put in 'Laxbro' neutral soap solution (MERCK) for 24 h. Then they were brushed thoroughly and washed in tap water twice and kept in hot air oven for 24 h. After taking out they were boiled in distilled water and again put in hot air oven for 24 h. Subsequently they were rinsed with double distilled water and covered with cotton and paper and autoclaved at 15 lb pressure for 20 minutes. The autoclaved materials were kept in hot air oven for 24 h and used for tissue culture work.

3.3.3 Preparation of media

Autoclaved double distilled water was used for reconstitution of media available in powder form. Dulbeco's modified Eagles medium (Hi Media), RPMI 1641 (Hi Media), and Medium 199 (Hi Media) were the three media employed. pH was adjusted at 7.2 after reconstitution. Foetal calf serum and goat serum filtered through Seitz filter was added at the level of 10 per cent. The following tissue culture media with serum supplementation were prepared and filtered through Seitz filter and

stored in the laboratory under refrigeration at 4°C. Trypsin (0.25%), Phosphate Buffered Saline and Hank's Balanced Salt Solution also were prepared and stored in the laboratory for tissue culture work. The media employed for the present study were as follows:

1. Dulbeco's modified Eagles Media (Hi Media) with 10% foetal calf serum
2. Dulbeco's modified Eagle's Media (Hi Media) with 10% goat serum
3. RPMI - 1640 (Hi Media) with 10% foetal calf serum
4. RPMI - 1640 (Hi Media) with 10% goat serum
5. Medium 199 (Hi Media) with 10% foetal calf serum
6. Medium 199 (Hi Media) with 10% goat serum

3.3.4 Cell dissociation

Fresh tumour tissue collected and washed a couple of times in HBSS with antibiotic was cut into small pieces with a sterile scissor and half of it was transferred to a 250 ml flask containing 100 ml of 0.25 per cent trypsin in PBS with antibiotics. Teflon coated magnetic stirring paddle was put in the flask and subjected to stirring for 10 minutes. The supernatent was poured off and fresh 100 ml 0.25 per cent Trypsin in PBS were added. Again subjected for stirring for 45 minutes. Serum (5 ml) was added to the suspension to neutralise the

trypsin The suspension was seived through a double layer of sterile muslin cloth into a sterile conical flask. The suspension was centrifuged at 1000 rpm for 5 minutes and the supernatant was poured off. The sediment rich in cells was washed with fresh HBSS and seeded into the tissue culture bottles, with about 20 ml of media supplemented with antibiotic at the following level.

Penicillin 200 IU/ml

Streptomycin 150 μg/ml

Gentamycin 50 μg/ml

Nystatin 100 IU/ml

The viable cell concentration was adjusted to 1.0×10^7 cell/ml. All the above procedures were done in sterile atmosphere in a laminar flow hood. Tightly capped bottles were kept in incubator set at 37°C for observation.

3.3.5 Explantation method

The tumour tissue collected in HBSS with antibiotics was cut into small pieces with a sterile scissor and washed twice in HBSS. They were further cut into small pieces with a sterile scalpel blade, and were washed twice in HBSS with antibiotic and each piece was placed in tissue culture bottle using a sterile pasteur pipette. Eight to ten such pieces were kept in a tissue

culture bottle. One drop of the medium was put on each piece and the tightly capped tissue culture bottles were placed in the incubator adjusted at 37°C for one hour to allow the tissue pieces to adhere to the glass surface. After one hour about 20 ml of media supplemented with antibiotics were poured into the tissue culture bottles. Tissue culture bottles were tightly capped under aseptic conditions and kept in horizontal position in the incubator set at 37°C.

3.3.6 Control

'Vero' cell line received from the National Facility for Animal Cell and Tissue Culture, Pune were maintained in the laboratory as control in Medium - 199 (Hi-Media) with 10 per cent foetal calf serum.

3.4 Staining techniques (H and E)

Tumour tissue was collected in 10 per cent buffered neutral formaldehyde. Routinely processed tissues were embedded in paraffin and sections cut at 5-6 μ thickness were stained with Harris Haematoxylin and Eosin (Luna, 1968).

3.5 AgNOR staining

Tumour tissue was collected in 10 per cent buffered neutral formaldehyde. Tissues were processed in the routine way and paraffin sections cut at 3-4 μ thickness were stained as

described by Egan and Crocker (1988). Four slides were prepared from each tumour tissue.

Stock solution

1. 50 per cent aqueous silver nitrate
2. 2 per cent gelatin in 1 per cent aqueous formic acid

Working solution

Solution No.1 and 2 were mixed at the ratio of 2:1.

Staining procedure

1. Sections were dewaxed in xylene.
2. Hydrated through descending grades of ethanol to deionised distilled water.
3. The final working solution was poured over the section and left for 60 minutes under safe light conditions at room temperature.
4. Sections were washed in deionised water.
5. Counter stained with Mayer's Haematoxylin.
6. Passed through ascending grades of ethanol and cleared in xylene and mounted in DPX mountant.
7. Counted the number of black spots in 100 nucleus under oil immersion objective of the microscope.

3.6 Mitochondrial staining

Method described by Takaya (1967) was followed. Tumour tissue was collected in 10 per cent buffered neutral formaldehyde as in the previous case and paraffin blocks were prepared. Sections cut at 2-5 μ thickness were stained as described below.

Solutions

1. Luxol fast blue 1 g dissolved in 1000 ml of 95% alcohol and 10 ml of 5% acetic acid were added.
2. 0.05% aqueous lithium carbonate
3. 0.5% aqueous phloxine
4. 5% aqueous phosphotungstic acid

Staining procedure

1. Sections were deparaffinised in xylene.
2. Washed in 3 changes of absolute alcohol.
3. Sections were incubated at 56°C for 6 h in luxol fast blue solution.
4. Passed through 95% alcohol.
5. Washed in distilled water.
6. Differentiated in lithium carbonate solution for 10 seconds.
7. 3 changes in 70% alcohol.
8. Washed in distilled water.
9. Stained with aqueous phloxine solution for 2-3 minutes.

10. Differentiated in phosphotungstic acid solution for two minutes.

11. Washed in running tap water for 5 minutes.

12. Dehydrated, cleared and mounted in DPX mountant.

3.7 Staining for GGT

Method described by Rutenburg et al. (1968) was followed. Small pieces of tumour tissue was collected in chilled acetone and fixed for 2-12 h. Rinsed in acetone 2 h, Benzene 3 changes for 15 minutes each, and in paraffin benzene mixture, 15 minutes and in paraffin 3 changes 15 minutes each and embedded in paraffin and blocks were made. Sections were cut at 4-5 micron thickness.

Solutions

Stock solution: 5 mg of GMNA in 0.1 ml of Dimethyl sulfoxide, 0.1 ml of 1 N NaOH and 1.8 ml of distilled water

Working solution

GMNA (2.5 mg/ml)	-	2 ml
Tris Buffer 0.1 M (pH 7.4)	-	10 ml
Normal saline	-	28 ml
Glycyl glycine	-	20 mg
Fast blue BBN	-	20 mg

Staining procedure

1. Sections were deparaffinised in xylene.
2. Hydrated through descending grades of ethanol to distilled water.
3. Incubated the sections in working solution for 30 minutes at 37°C.
4. Rinsed in normal saline for 2 minutes.
5. Rinsed in 0.1 M cupric sulphate solution for 2 minutes.
6. Rinsed in normal saline for 2 minutes.
7. Rinsed in water.
8. Counter stained in Harris Haematoxylin
9. Dehydrated through ascending grades of alcohol.
10. Cleared in xylene and mounted with DPX mountant.

3.8 Serum copper and zinc estimation

Glasswares used for collection of blood for harvesting serum was washed thoroughly and rinsed with deionised water and dried, by keeping in hot air oven overnight. Serum was harvested with utmost care to avoid haemolysis and collected in stoppered bottles and stored at $^{-}$70°C. The serum copper and zinc levels were estimated by atomic absorption spectophotometry, using Perkin-Elmer 2380 atomic absorption spectrophotometer (Makino and Takahara, 1981). Serum from 12 healthy cows from

the University Livestock Farm, Mannuthy also were analysed as control.

3.9 Parentage of sinus tumour animals

Data regarding the parentage of sinus tumour animals were collected from the livestock register maintained at the University Livestock Farm, Mannuthy.

3.10 Ultrastructural study

Cells from tissue culture were pelletted and fixed in 2.5 per cent gluteraldehde in cacodylate buffer. Subsequently they were post fixed in osmium tetroxide. The material was embedded in spurr. Sections were cut with glass knives on LKB ultratome. Thin sections were picked up on copper grids and examined with Hitachi-H-600 A electron microscope at 50 KV.

Results

RESULTS

4.1. Incidence and distribution

Nasal smears were received from various parts of Kerala and Military Farm Pune, for exfoliative cytology study and confirmatory diagnosis of ethmoid carcinoma. During the period of the study from January 1989 to December 1991, 519 smears were received for cytological examination. Out of the 519 smears, 9 smears were from Military Farm, Pune and 510 smears from different parts of Kerala. During the year 1989, 182 smears were received for examination, out of which 110 smears were found positive for ethmoid carcinoma. In the year 1990, 176 smears were presented and 87 were found positive. During 1991, 161 smears were submitted and 97 were declared positive for ethmoid carcinoma. In all, out of the 519 smears received for examination 294 were found positive for ethmoid carcinoma.

4.1.1 Month-wise distribution

During the year 1989, 182 smears were presented for cytological examination. Among the smears presented 60.43 per cent were positive for ethmoid carcinoma. Highest percentage of presented cases (15.93%) was during March and the lowest was during January (4.94%).

Among the positive cases, highest number of positive cases was recorded during March, July and August (12.73%) and the month of January and September showed the least percentage of positive cases (4.54%) (Table 1). In the year 1990, out of the 176 smears submitted only 49.43 per cent of the smears were declared positive. During this year also maximum number of smears presented for examination was in March (13.64%), and the lowest was during August (5.11%). Out of the positive cases, highest percentage of positive cases was during January (14.94%) and the lowest percentage of positive cases (4.6%) was in June and July (Table 2). In the year 1991, out of the 161 smears presented 60.25 per cent of the cases were found positive for ethmoid carcinoma. During this year, highest percentage of record of incidence was during October (16.14%) and the lowest was in December (3.10%). Among the positive cases also the month of October showed highest percentage (19.58%) and the month of July and December recorded the lowest percentage of incidence (3.09%) (Table 3).

During the entire period of the study, out of the 519 nasal smears presented for examination, 56.63 per cent of the smears were found positive for ethmoid carcinoma. Highest percentage of submission of samples was during March (12.72%), and the lowest percentage of cases submitted for examination was during February and September (6.36%). Among the positive cases, highest percentage was during March (12.92%) and the lowest was during June and September (5.78%) (Table 4 and Fig.1).

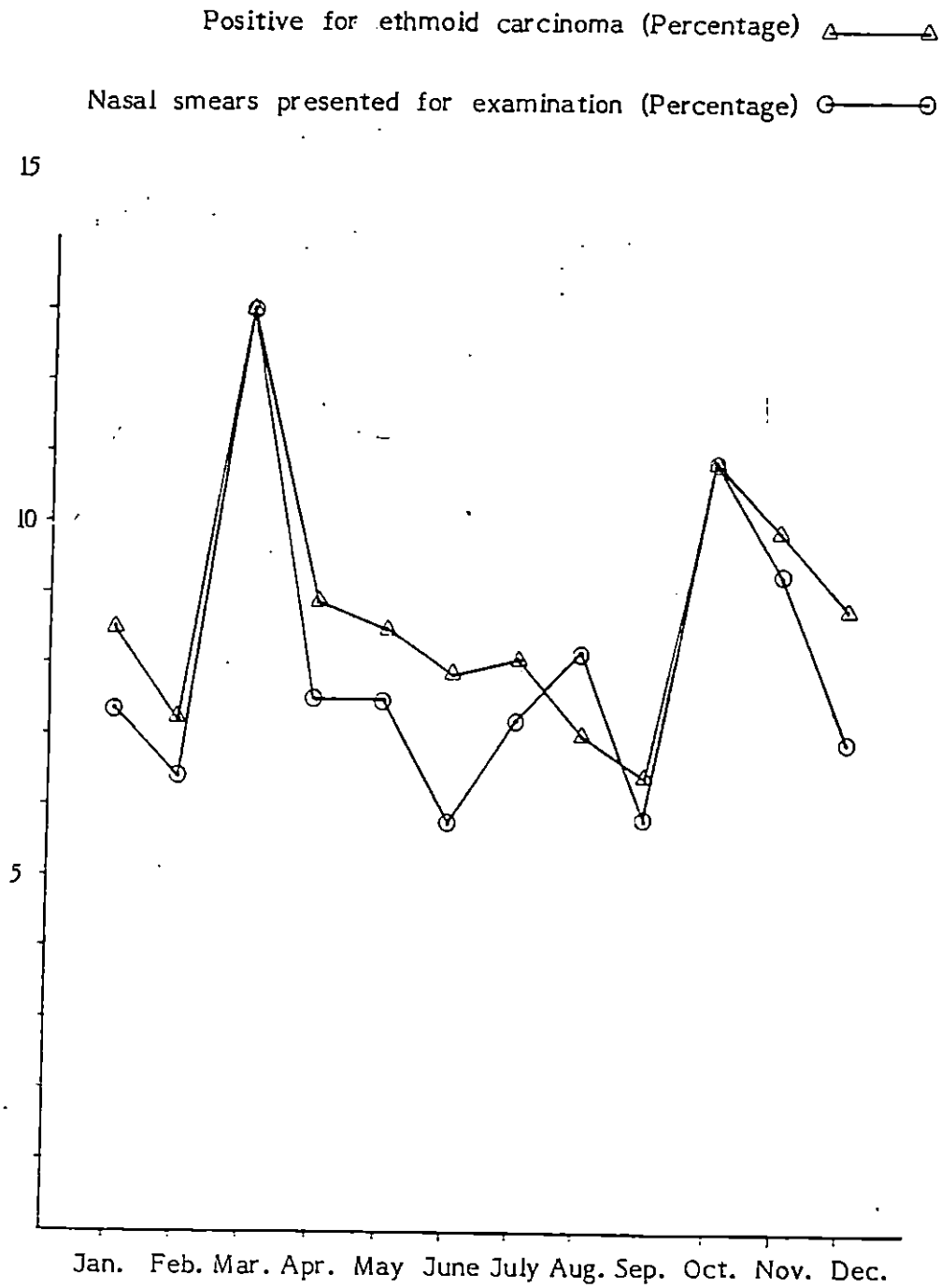


FIG.1 MONTH-WISE DISTRIBUTION OF NASAL SMEARS PRESENTED AND POSITIVE CASES (PERCENTAGE-WISE) OF ETHMOID CARCINOMA

4.1.2 District-wise distribution

During the year 1989, 178 smears were received for examination. Highest percentage of report was from Thrissur district (32.02%) and the lowest was from Kasaragod district (8.00%). Among the positive cases, maximum percentage of cases was from Thrissur district and the lowest was from Kasaragod and Kozhikode districts (0%) (Table 5). In the year 1990, 171 smears were received. Highest percentage of report was from Thrissur (36.84%) and the lowest was from Thiruvananthapuram, Kannur and Kasaragod (0%). Among the positive cases also Thrissur district ranked first (39.53%) and Thiruvananthapuram, Malappuram, Kannur and Kasaragod districts were ranked, the lowest (0%) (Table 6). In the year 1991, 161 smears were received, out of this, highest percentage was from Thrissur district (44.72%) and the lowest was from Kannur (0%). Among the positive cases also, Thrissur district recorded the highest percentage of report (43.3%) and Kannur reported the lowest percentage (0%) (Table 7). Among the 510 smears received for examination, during the entire period of study 37.65 per cent was from Thrissur district and the lowest was from the districts of Kannur and Kasaragod (0.39%). Among the positive cases also Thrissur district recorded the highest percentage of report (21.57%) and Kasaragod district recorded the lowest percentage of report (0.35%) (Table 8 and Fig.2).

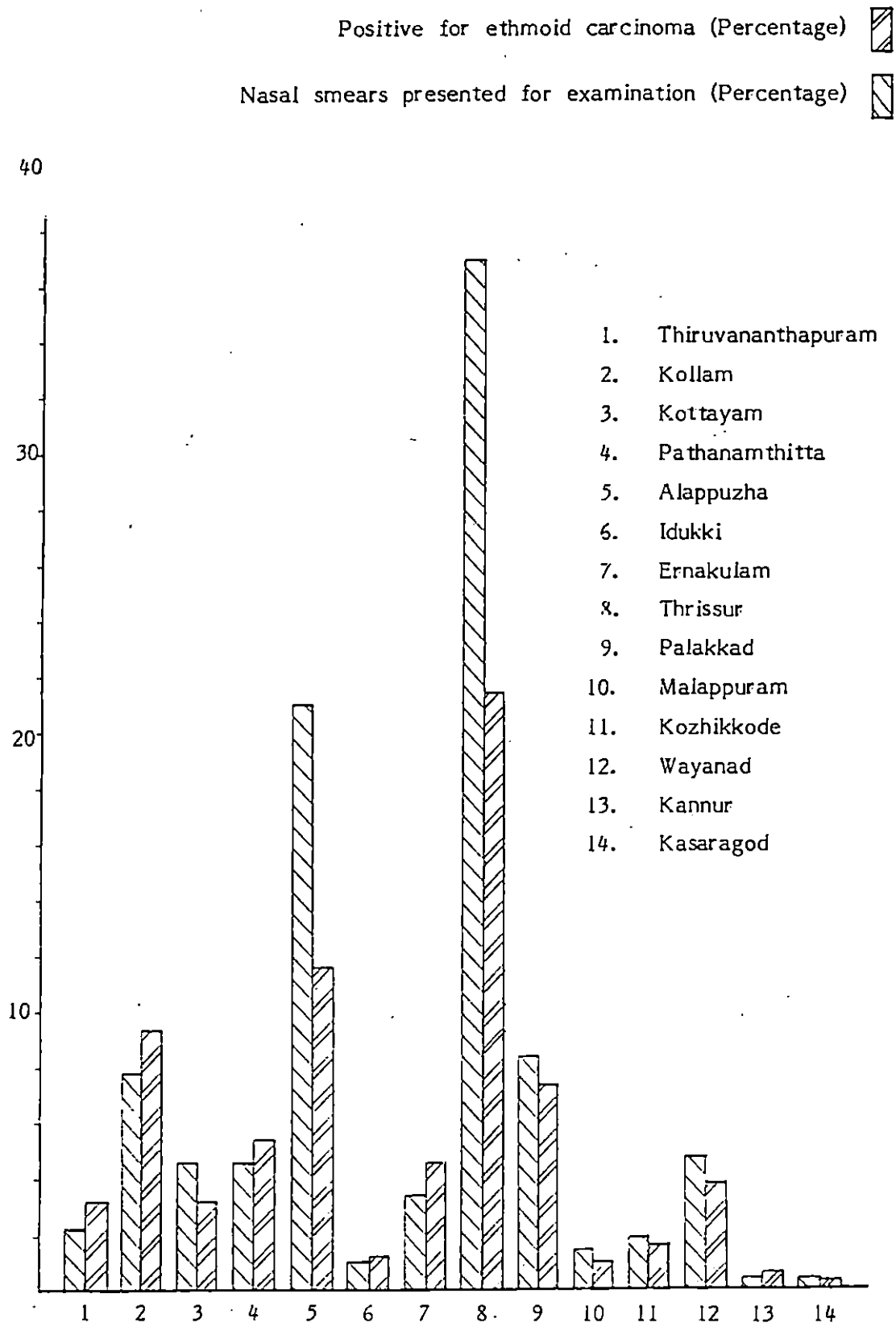


FIG.2 DISTRICT-WISE DISTRIBUTION OF NASAL SMEARS EXAMINED AND POSITIVE CASES OF ETHMOID CARCINOMA

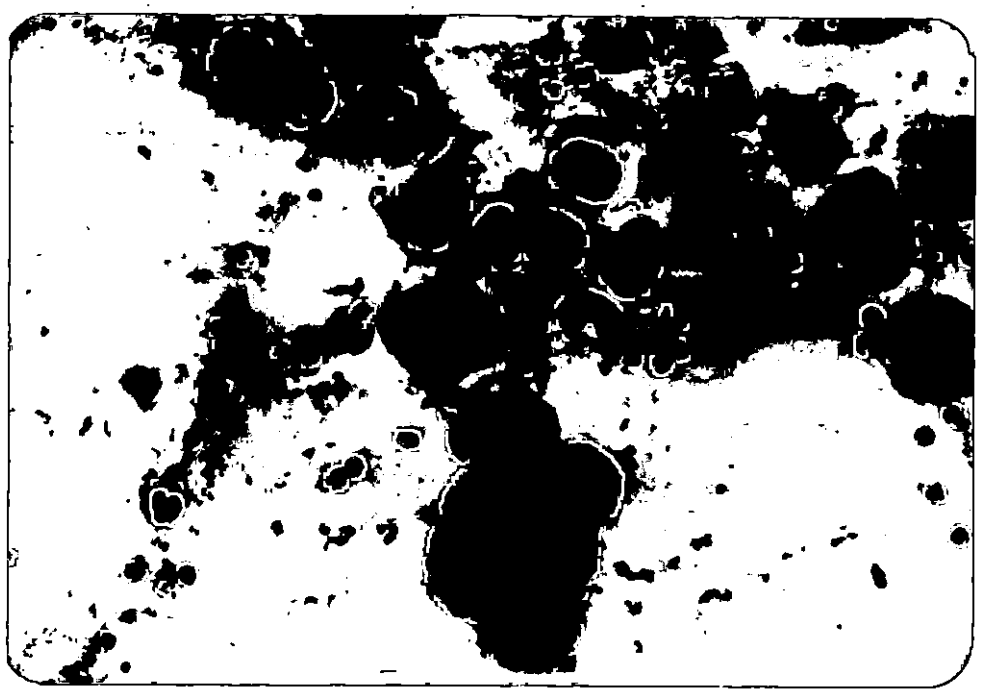
4.2 Exfoliative cytology and histopathology

Nasal smears of suspected tumour bearing animals received were examined for exfoliated neoplastic cells (519). Smears were examined for exfoliated neoplastic cells employing Wright's staining technique. Hyperchromatic condensed nucleus, basophilic cytoplasm with reduced nuclear cytoplasmic ratio, anisokaryosis and anisocytosis were considered as characteristics of cells positive for ethmoid carcinoma (Fig.3). After confirming the diagnosis as ethmoid carcinoma, the animals were procured and maintained for the study. They belonged to the University Livestock Farm, Mannuthy (6), Cattle Breeding Farm, Thumboormuzhi (1), and Private owners in and around Thrissur (16) (Table 9). These animals were euthanised and examined for the tumour mass in the ethmoid region, except in four cases in which animals died unexpectedly before euthanesia. In all the cases detailed post-mortem examination was also conducted. All the animals showed tumour mass in the ethmoid area (Fig.4). On histopathological examination 14 cases were diagnosed as adenocarcinoma (Fig.5), two cases as papillary adenocarcinoma (Fig.6), three cases as squamous cell carcinoma (Fig.7), two cases as transitional cell carcinoma (Fig.8) and two as undifferentiated carcinoma (Fig.9).

Histopathologically, adenocarcinoma showed definite pattern of acini formation. Neoplastic cells were seen proliferating and formed many irregular layers of cells in the

Fig.3 Nasal smear stained with Wright's stain, positive for ethmoid carcinoma. Individual and sheets of neoplastic cells are seen x 1000

Fig.4 Tumour mass in the ethmoid region extending and filling the nasal chamber



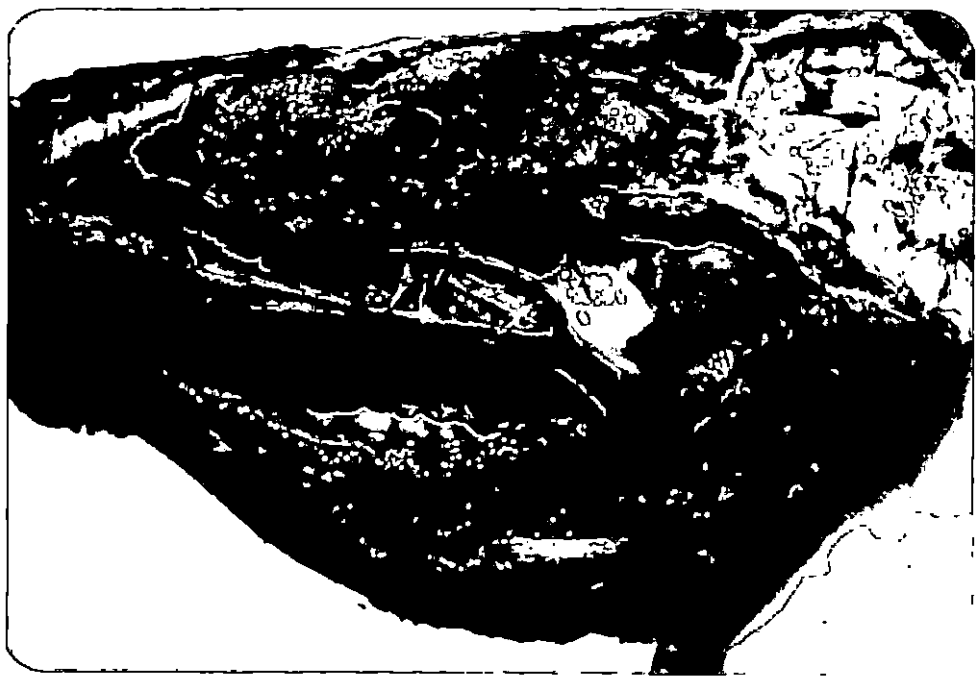


Fig.5 Adenocarcinoma - Acinar pattern widely thick fibro collagenous stroma H&E x 250

Fig.6 Papillary adenocarcinoma - Papillary projections - Core of the papillary projections formed by the fibrous tissue H&E x 250

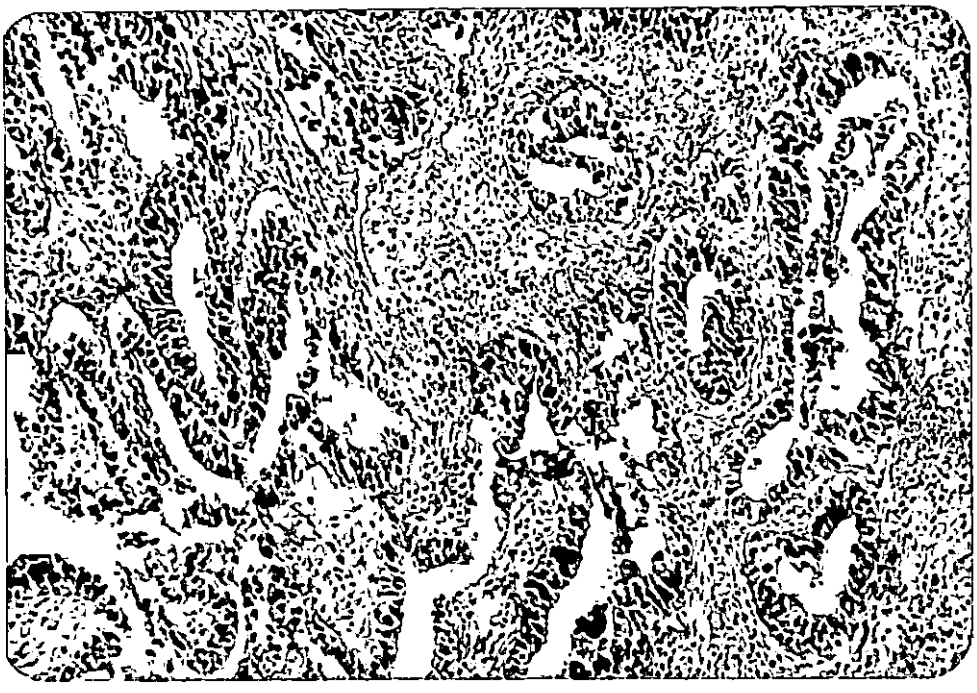
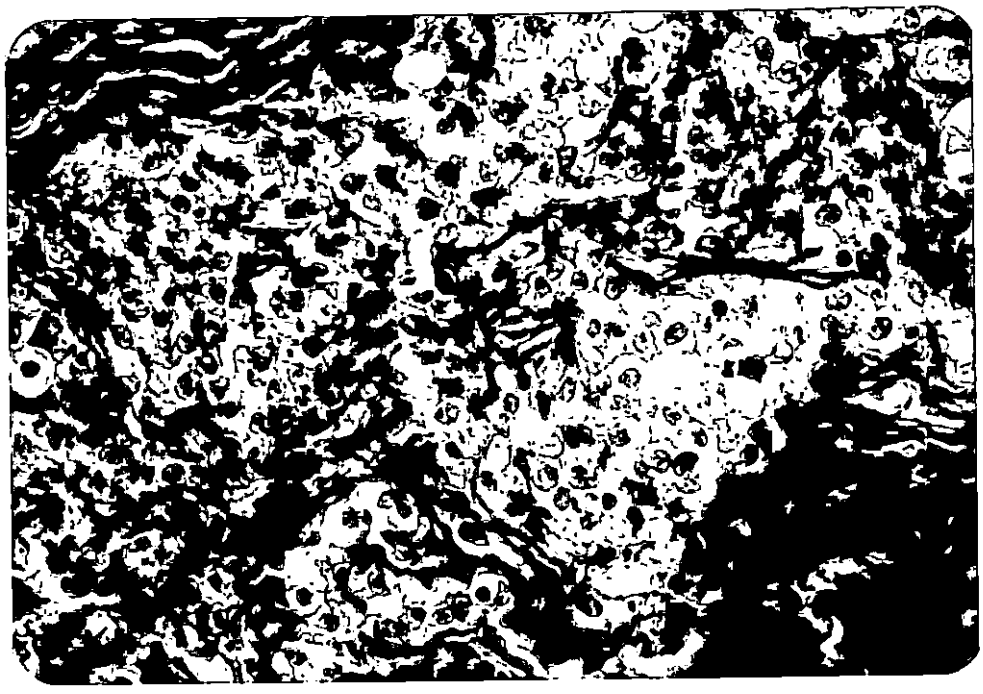


Fig.7 Squamous cell carcinoma - Keratinizing squamous cells H&E x 250

Fig.8 Transitional cell carcinoma - Cells of various morphology ranging from columnar to squamous and intermediate stages H&E x 250



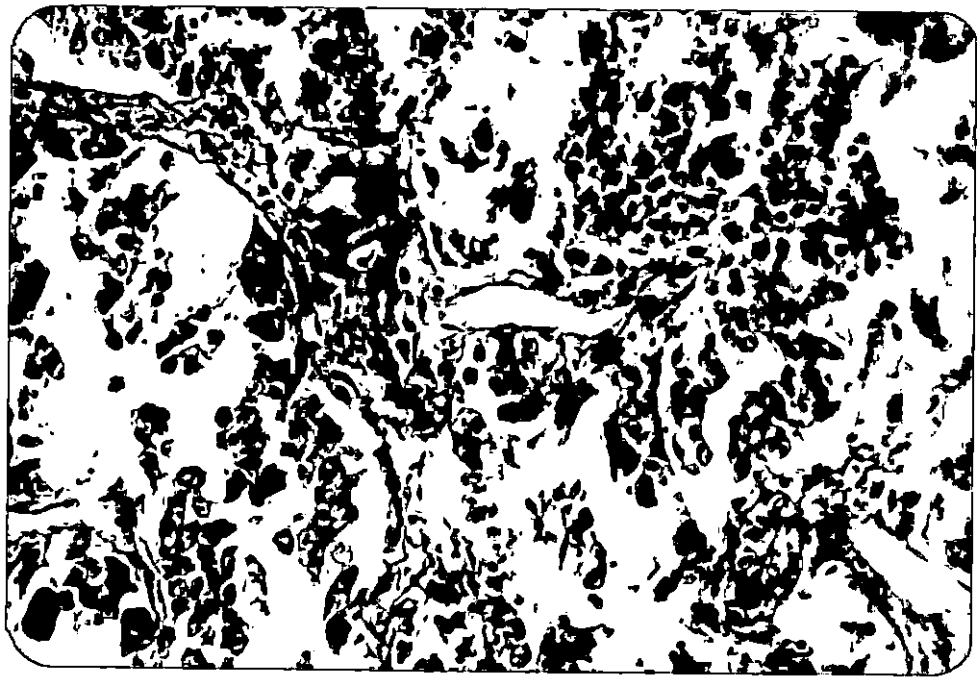
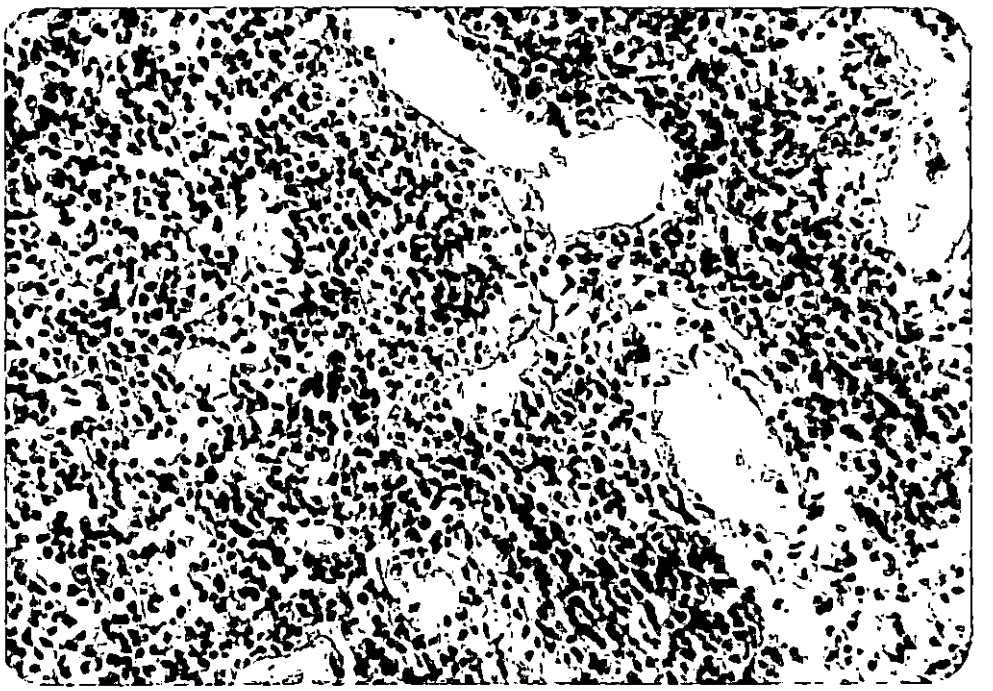
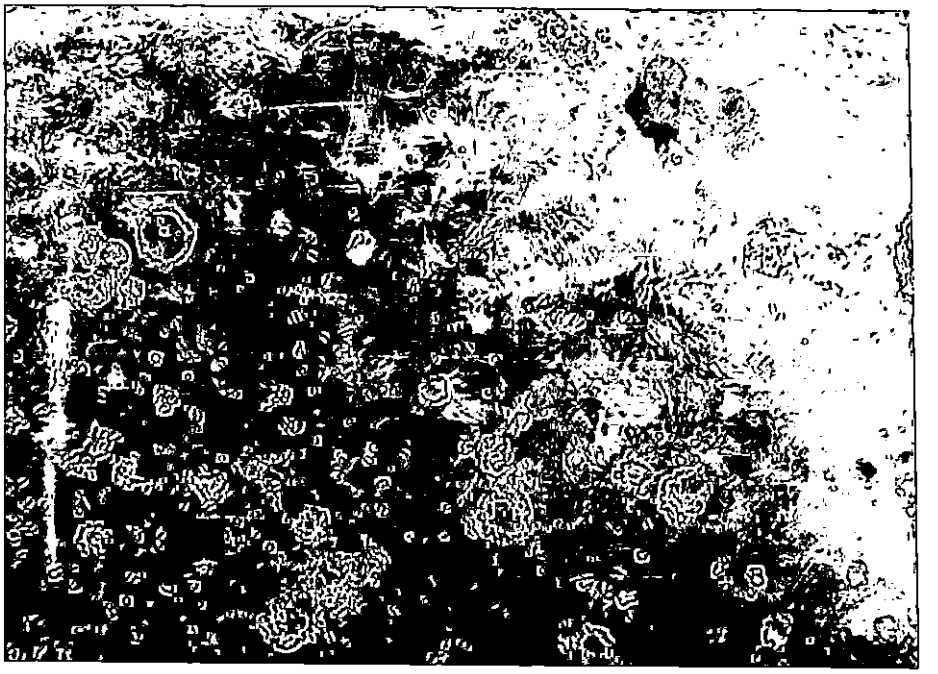


Fig.9 Undifferentiated carcinoma - Undifferentiated uniform round cells arranged in groups H&E x 160

Fig.10 Carcinoma cells in culture (RPMI-1640) 4 days- clumps of cells and individual cells x 250





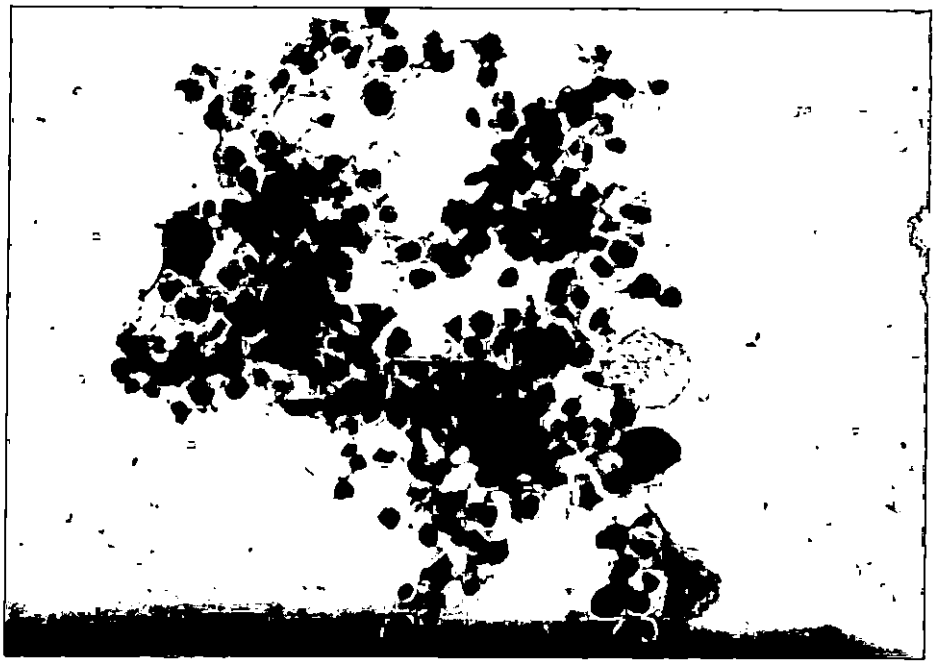
acini. Desmoplastic reaction was very prominent. Papillary adenocarcinoma showed finger like projections. The core portion of the finger like processes comprised of fibrous tissue and neoplastic cells were seen proliferating towards the periphery. In squamous cell carcinoma, there was neoplastic cells of squamous epithelial cell morphology. Keratin, a product of these cells was seen in the centre of the collection of cells giving an appearance of cell nests. In transitional cell carcinoma the neoplastic cells were seen in different morphology varying from columnar to squamous type and in various stages of tranformation. In undifferentiated carcinoma, the neoplastic cells were poorly differentiated and appeared like a clump of cells. The cells were anaplastic and had no definite architectural pattern.

4.3 Cell culture study

Tumour tissue was collected from nineteen cases for cell culture study. In vitro culture of tumour cells was tried using Medium - 199 (Hi Media), Dulbeco's modified Eagles medium (Hi Media) and RPMI - 1640 (Hi Media) with either 10 per cent foetal calf serum or 10 per cent goat serum. Viable cell count made employing trypan blue dye exclusion technique showed increased number of dead cells in most of the cases. Many of the cultures especially explantation cultures showed bacterial and fungal contamination, in spite of using antibiotic and antifungal agents at the maximum permissible level. In five cases, the cells seeded in the bottle remained in suspension

Fig.11 Carcinoma cells in culture (RPMI-1640) 13 days - confluent monolayer x 160

Fig.12 Smear of cells of ethmoid carcinoma from culture - Geimsa stain x 1000



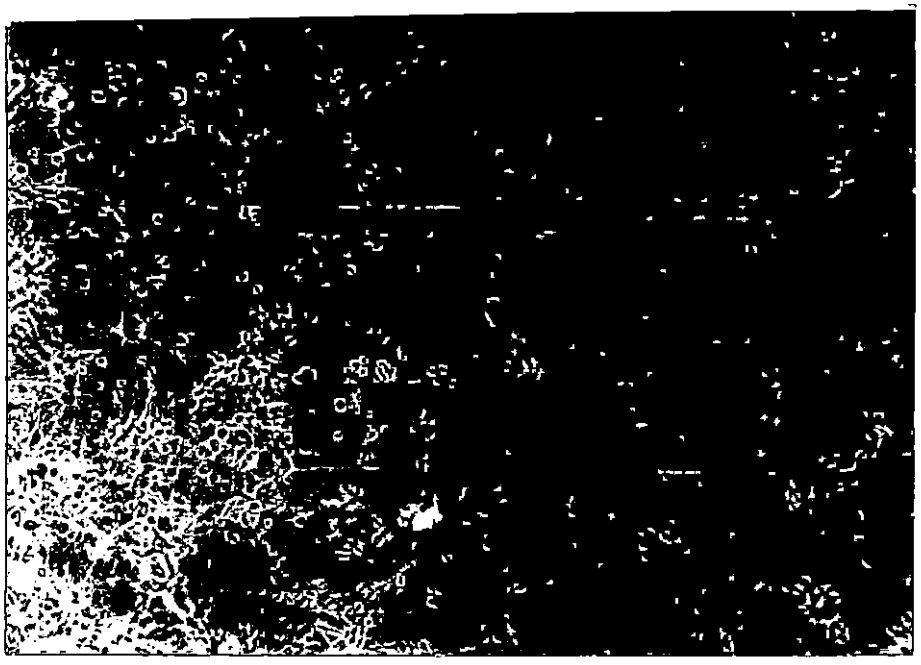
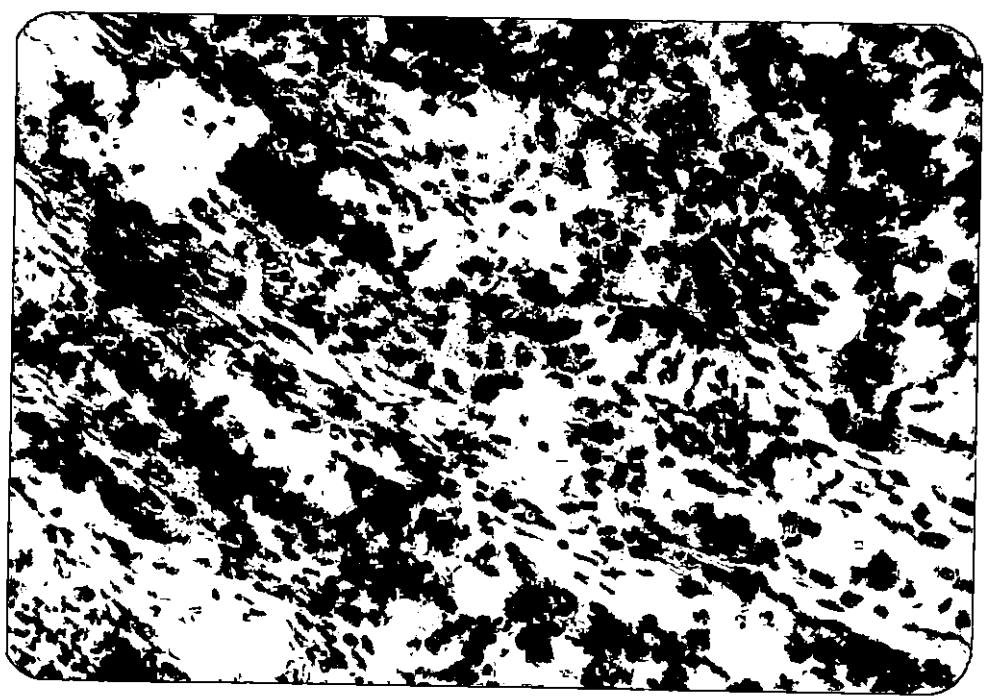


Fig.13 Electron micrograph of ethmoid carcinoma cell x 12,000

Fig.14 'Vero' cell line in Medium - 199 confluent monolayer x 160

Fig.15 Gamma glutamyl transpeptidase activity in ethmoid carcinoma. Cytoplasm of the tumour cells showing enzyme activity. Counter stain - Haematoxylin x 250



without adhering to the glass surface, and there was no contamination also. Explantation cultures also remained without contamination, but without any tendency for growth or proliferation. In the 18th attempt with tumour tissue collected from a cow showed active monolayer formation and proliferation (Fig.10). The media employed in this case was RPMI-1640 with 10 per cent foetal calf serum. Histologically the tumour was diagnosed as adenocarcinoma. The cell layer became confluent within 13 days (Fig.11). The monolayer was dissociated using trypsin 0.25 per cent in PBS and seeded in two bottles each (First Passage). Those cells were also seen adhering to glass surface and dividing. Smear prepared from the cell suspension for seeding in the first passage was stained with Wright's stain or Geimsa showed large cells with well defined hyperchromatic nucleus and distinct cytoplasm (Fig.12). The nucleus was oval or round. Cells in various stages of division were also seen in the smear.

The ultrastructural studies also revealed the epithelial nature of the neoplastic cell (Fig.13). The cells varied in size and shape. Generally the plasma membranes were irregular and ruffled. The nuclei showed great irregularity in size and shape. In general, enchromatin was predominant and heterochromatin could be seen as small aggregates. The mitochondria were round or oval and some of them were seen associated with endoplasmic reticulum.

As a control 'vero' cell line was maintained in Medium-199 supplemented with 10 per cent foetal calf serum (Fig.14).

4.4 Histochemical demonstration of GGT

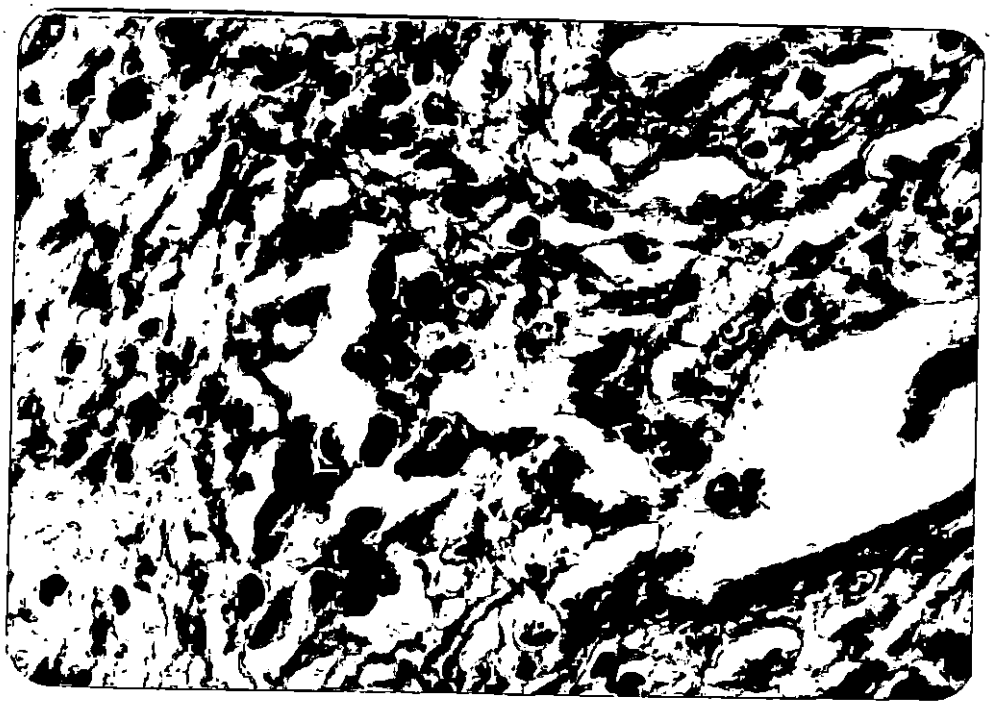
Two sections each from twelve cases were stained for GGT. It comprised of one case of papillary adenocarcinoma, nine cases of adenocarcinoma and two cases of squamous cell carcinoma. Tissue sections stained for the demonstration of GGT activity showed positive reaction. Enzyme activity was predominent in the cytoplasm of tumour cells. The stromal cells and interstitial tissue showed no indication of GGT (Fig.15). Normal ethmoid epithelium stained as control also did not show the evidence of GGT. There was no difference in the expression of GGT activity in the different histological types of tumour.

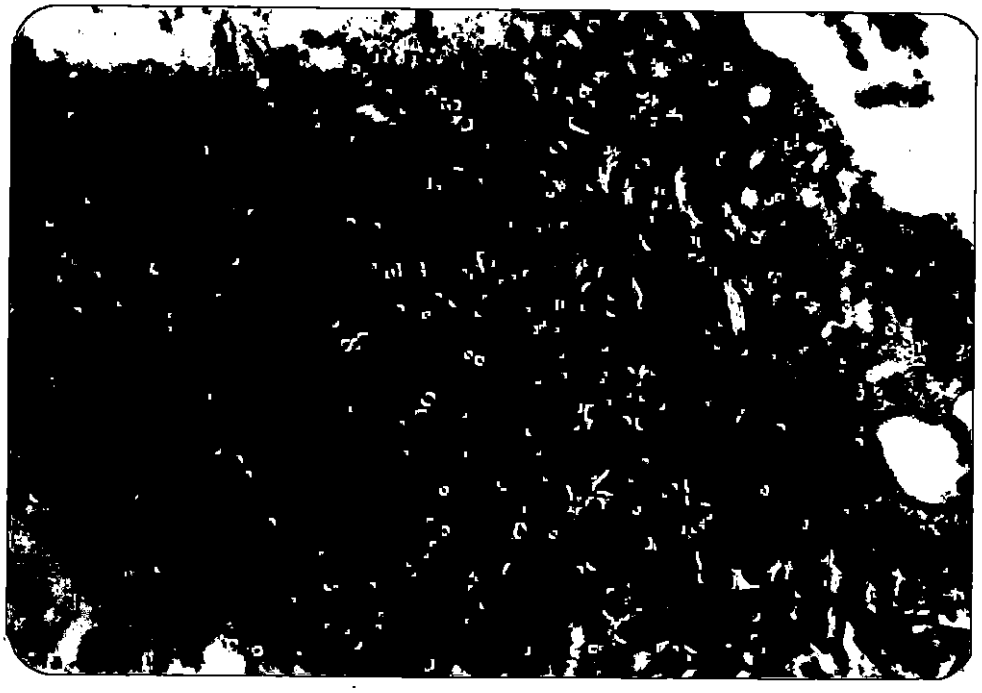
4.5 Histochemical demonstration of mitochondria

Two sections each from twelve cases were stained for mitochondria. It consisted of one case of papillary adenocarcinoma, nine cases of adenocarcinoma and two cases of squamous cell carcinoma. The cytoplasm of the tumour cells showed diffuse greenish colour and some cells with granules of various sizes in the cytoplasm were also noticed, indicating pronounced mitochondrial proliferation and/or damage to the mitochondria (Fig.16). There was no difference in the staining pattern in different histological types of tumour. Normal ethmoid mucosa was also stained for mitochondria as a control.

Fig.16 Luxol fast blue staining for mitochondria in ethmoid carcinoma cells. Nuclei stained red and mitochondria green x 250

Fig.17 Nucleolar organiser regions - Dark round dots in the nucleus x 400





4.6 Histochemical demonstration of AgNOR

Histological sections from 14 cases were analysed for AgNOR count (Fig.17). Among the 14 cases, histopathologically 12 were diagnosed as adenocarcinoma and two as squamous cell carcinoma. Four sections each from one case was counted for AgNOR. AgNOR appeared as black round dots in the nucleus. The size and number of AgNOR's showed wide variation. As the number of AgNOR's increase, there was comparative reduction in the size of AgNOR. Among the adenocarcinoma cases, the average AgNOR count ranged from 1.24 ± 0.07 to 2.22 ± 0.09. The average AgNOR count for adenocarcinoma was 1.79 ± 0.08. The AgNOR count in squamous cell carcinoma ranged from 1.81 ± 0.31 to 2.29 ± 0.19 and the average was 2.05 ± 0.24 (Table 10 and Fig.18).

4.7 Serum zinc and copper levels

Serum zinc and copper level of 19 tumour bearing animals were examined. The average serum zinc level was 0.99 ± 0.11 ppm. Serum from 12 apparently healthy cows from the University Livestock Farm, Mannuthy was analysed for serum zinc level. The average serum zinc level in healthy animals was 1.35 ± 0.23 ppm (Table 11). Serum copper level in normal healthy animals was 1.04 ± 0.07 ppm. Serum copper level in tumour bearing animals was 0.86 ± 0.06 ppm (Table 12). There was reduction in serum zinc and copper level in all the tumour bearing animals when

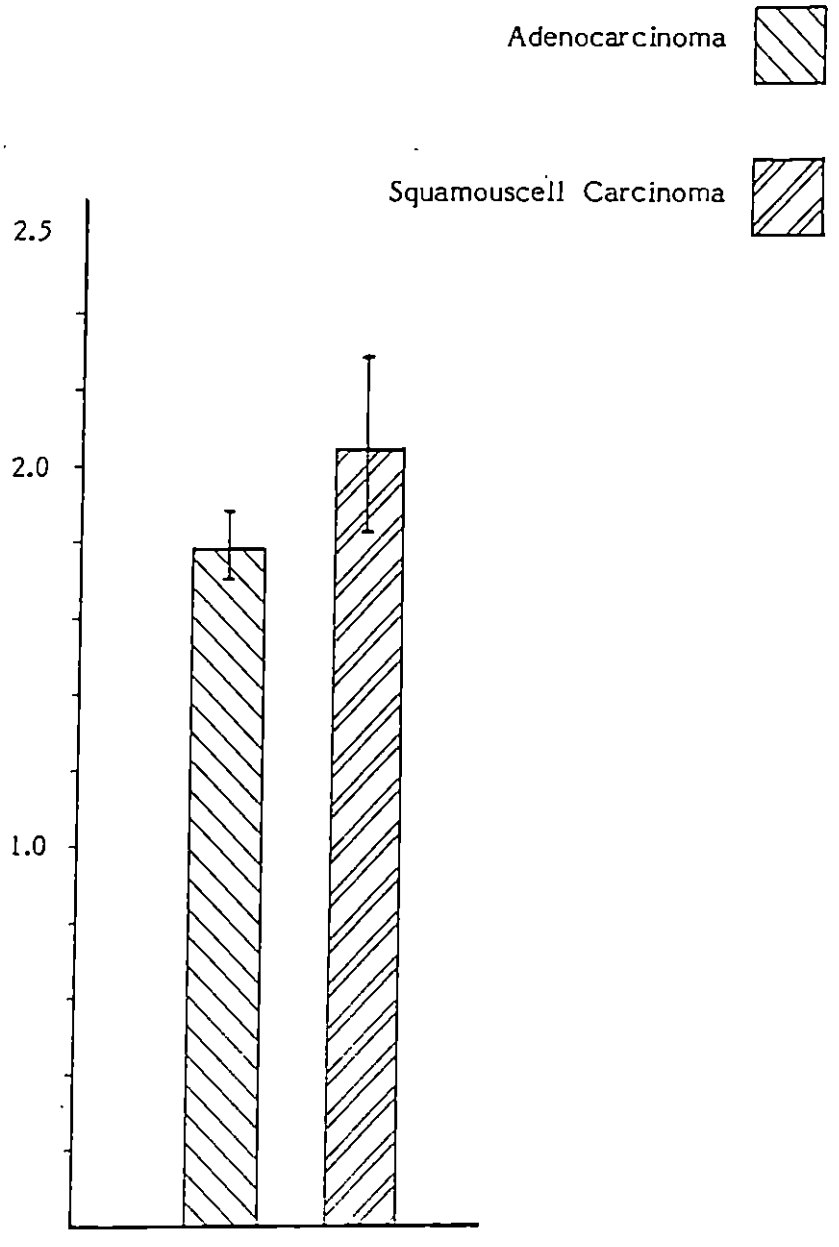


FIG.18 AgNOR COUNT IN ETHMOID CARCINOMA

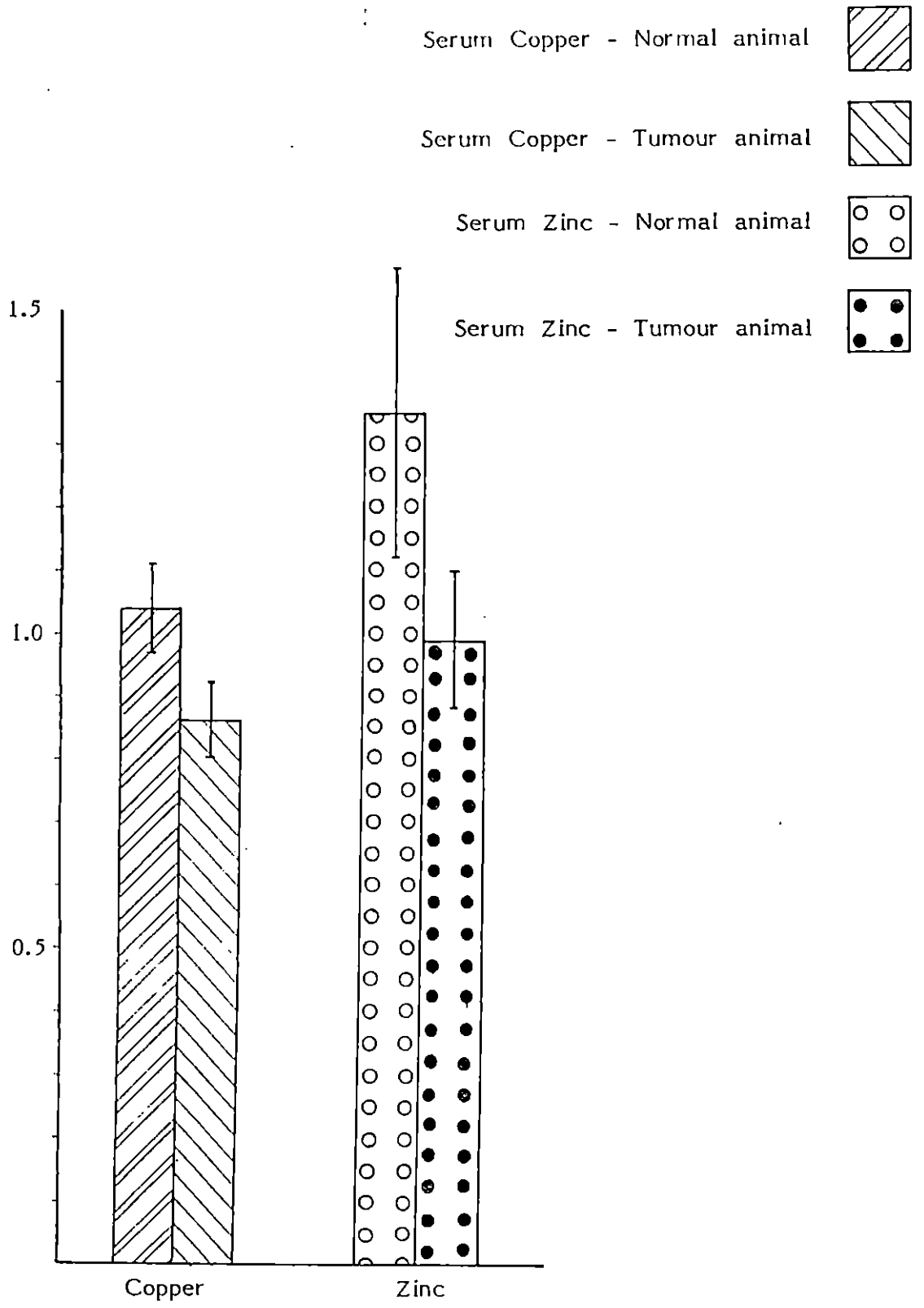


FIG.19 SERUM COPPER AND ZINC LEVEL IN TUMOUR BEARING AND NORMAL NON TUMOUR BEARING ANIMALS

compared to the controls (Fig.19). However, the reduction in serum zinc and copper level was not statistically significant.

4.8 Parentage of ethmoid carcinoma animals

Parentage of six ethmoid carcinoma animals received from the University Livestock Farm, Mannuthy was screened to find out genetic predisposition, if any. It showed that, all the animals were cross-bred, and sire of two ethmoid carcinoma animals were the same (Table 13). Assessment of parentage of sires and dams of ethmoid carcinoma animals revealed no significant information.

Table 1. Month-wise distribution of nasal smears examined during 1989

Month	Presented	Positive	Negative	% of Presented	% of Positive	% of Negative
January	9	5	4	4.94	4.54	5.55
February	13	5	8	7.14	4.54	11.11
March	29	14	15	15.93	12.73	20.83
April	12	6	6	6.59	5.45	8.35
May	15	9	6	8.24	8.18	8.33
June	15	6	9	8.24	5.45	12.50
July	17	14	3	9.34	12.73	4.17
August	18	14	4	9.89	12.73	5.35
September	10	5	5	5.49	4.54	6.94
October	13	7	6	7.14	6.36	8.33
November	15	12	3	8.24	10.91	4.17
December	16	13	3	8.79	11.82	4.17
Total	182	110	72		60.43	39.57

Table 2. Month-wise distribution of nasal smears examined during 1990

Month	Presented	Positive	Negative	% of Presented	% of Positive	% of Negative
January	19	13	6	10.79	14.94	6.74
February	10	7	3	5.68	8.05	3.37
March	24	16	8	13.64	8.39	8.99
April	13	6	7	7.39	6.90	7.86
May	17	7	10	9.66	8.05	11.24
June	14	4	10	7.95	4.60	11.24
July	12	4	8	6.82	4.60	8.99
August	9	5	4	5.11	5.75	4.99
September	11	4	7	6.25	4.67	7.56
October	17	6	11	9.66	6.90	12.36
November	15	5	10	8.52	5.75	11.24
December	15	10	5	8.52	11.49	5.62
Total	176	87	89		49.43	50.57

Table 3. Month-wise distribution of nasal smears examined during 1991

Month	Presented	Positive	Negative	% of Presented	% of Positive	% of Negative
January	10	7	3	6.21	7.22	4.69
February	10	9	1	6.21	9.28	1.56
March	13	8	5	8.07	8.25	7.81
April	21	10	1	13.04	10.31	17.19
May	12	6	6	7.45	6.19	9.37
June	12	7	5	7.45	7.22	7.81
July	13	3	10	8.07	3.09	15.62
August	9	5	4	5.59	5.15	6.25
September	12	8	4	7.45	8.25	6.25
October	26	19	7	16.14	19.58	10.94
November	18	12	6	11.18	12.37	9.37
December	5	3	2	3.10	3.09	3.13
Total	161	97	64		60.25	39.75

Table 4. Month-wise distribution of nasal smears examined during January 1989 to December 1991

Month	Presented	Positive	Negative	% of Presented	% of Positive	% of Negative
January	38	25	13	7.32	8.50	5.77
February	33	21	12	6.36	7.14	5.33
March	66	38	28	12.72	12.92	12.44
April	46	22	24	8.86	7.48	10.66
May	44	22	22	8.48	7.48	9.77
June	41	17	24	7.90	5.78	10.66
July	42	21	21	8.09	7.14	9.33
August	36	24	12	6.94	8.16	5.33
September	33	17	16	6.36	5.78	7.11
October	56	32	24	10.79	10.88	10.66
November	48	29	19	9.25	9.86	8.44
December	36	26	10	6.94	8.84	4.44
Total	519	294	225		56.65	43.35

Table 5. District-wise distribution of nasal smears examined during 1989

District	Presented	Positive	Negative	% of Presented	% of Positive	% of Negative
Thiruvananthapuram	8	6	2	4.49	5.66	2.78
Kollam	18	13	5	10.11	12.26	6.94
Kottayam	9	5	4	5.06	4.72	5.56
Pathanamthitta	7	3	4	3.93	2.83	5.56
Alapuzha	31	20	11	17.42	18.87	15.28
Idukki	2	2	0	1.12	1.89	0.00
Ernakulam	5	3	2	2.81	2.83	2.78
Thrissur	57	34	23	32.02	32.08	31.94
Palakkad	21	11	10	11.80	10.38	13.89
Malappuram	6	2	4	3.37	1.89	5.56
Kozhikode	2	0	2	1.12	0.00	2.78
Wynad	10	5	5	5.62	4.72	6.94
Kannur	2	2	0	1.12	1.89	0.00
Kasaragod	0	0	0	0.00	0.00	0.00
Total	178	106	72		59.55	40.45

* 4 smears presented from Pune and all were positive

Table 6. District-wise distribution of nasal smears examined during 1990

District	Presented	Positive	Negative	% of Presented	% of Positive	% of Negative
Thiruvananthapuram	0	0	0	0.00	0.00	0.00
Kollam	15	9	6	8.77	10.46	7.06
Kottayam	6	2	4	3.51	2.33	4.71
Pathanamthitta	11	8	3	6.43	9.30	3.53
Alapuzha	42	20	22	24.56	23.26	25.88
Idukki	2	1	1	1.17	1.16	1.18
Ernakulam	6	3	3	3.91	3.49	3.53
Thrissur	63	34	29	36.84	39.53	34.12
Palakkad	11	2	9	6.43	2.33	10.59
Malappuram	1	0	1	0.58	0.00	1.18
Kozhikode	5	3	2	2.92	3.49	2.35
Wynad	9	4	5	5.26	4.65	5.88
Kannur	0	0	0	0.00	0.00	0.00
Kasaragod	0	0	0	0.00	0.00	0.00
Total	171	86	85		50.29	49.71

* 5 smears presented from Pune, of which one was positive

Table 7. District-wise distribution of nasal smears examined during 1991

District	Presented	Positive	Negative	% of Presented	% of Positive	% of Negative
Thiruvananthapuram	3	3	0	1.86	3.09	0.00
Kollam	7	5	2	4.35	5.15	3.12
Kottayam	8	2	6	4.97	2.06	9.38
Pathanamthitta	5	5	0	3.10	5.15	0.00
Alapuzha	36	19	17	22.36	19.59	26.56
Idukki	1	0	1	0.62	0.00	1.56
Ernakulam	7	7	7	4.35	7.22	0.00
Thrissur	72	42	30	44.72	43.30	46.88
Palakkad	11	8	3	6.83	8.25	4.68
Malappuram	1	1	0	0.62	1.03	0.00
Kozhikode	3	2	1	1.86	2.06	1.56
Wynad	5	2	3	3.10	2.06	4.68
Kannur	0	0	0	0.00	0.00	0.00
Kasaragod	2	1	1	1.24	1.03	1.56
Total	161	97	64		60.25	39.75

Table 8. District-wise distribution of nasal smears examined during 1989 to 1991

District	Presented	Positive	Negative	% of Presented	% of Positive	% of Negative
Thiruvananthapuram	11	9	2	2.16	3.11	0.91
Kollam	40	27	13	7.84	9.34	5.88
Kottayam	23	9	14	4.51	3.11	6.34
Pathanamthitta	23	16	7	4.51	5.54	3.17
Alapuzha	109	59	50	21.37	11.57	22.62
Idukki	5	3	2	0.98	1.04	0.91
Ernakulam	18	13	5	3.53	4.50	2.26
Thrissur	192	110	82	37.65	21.57	37.10
Palakkad	43	21	22	8.43	7.27	9.96
Malappuram	8	3	5	1.57	1.04	2.26
Kozhikode	10	5	5	1.96	1.73	2.26
Wynad	24	11	3	4.71	3.81	5.88
Kannur	2	2	0	0.39	0.69	0.00
Kasaragod	2	1	1	0.39	0.35	0.45
Total	510	289	221		56.67	43.33

* 9 smears were received from Pune, of which 5 was positive

Table 9. Source of sinus tumour animals and data on histopathological diagnosis

Sl. No.	Source	Date of sacrifice/ death	Histopathological diagnosis
1.	University Livestock Farm, Mannuthy	28.03.1990	Papillary adenocarcinoma
2.	Mr. G. Karunakaran Kuthuparambil Puthenchira Mala	22.05.1990	Adenocarcinoma
3.	University Livestock Farm, Mannuthy	05.06.1990	Adenocarcinoma
4.	University Livestock Farm, Mannuthy	28.06.1990	Adenocarcinoma
5.	University Livestock Farm, Mannuthy	02.08.1990	Adenocarcinoma
6.	Mr. Raman Nair Near Paramekkavu Temple Thrissur	24.11.1990	Squamous cell carcinoma
7.	Mulamkunnathukavu Thrissur	01.03.1991	Squamous cell carcinoma
8.	Mrs. Madhura Meenakshi Alathur Palakkad Dist.	02.06.1991	Adenocarcinoma
9.	Leprosy Patient's Society, Koratty Thrissur	07.06.1991	Adenocarcinoma
10.	Mr. M.K. Unni Mathedathu, Nadathara Thrissur Dist.	18.06.1991	Adenocarcinoma
11.	University Livestock Farm, Mannuthy	29.06.1991	Adenocarcinoma

Contd.

Table 9 (Contd.)

Sl. No.	Source	Date of sacrifice/ death	Histopathological diagnosis
12.	University Livestock Farm, Mannuthy	16.07.1991	Adenocarcinoma
13.	Shri. P.N. Nambeesan Puzhpakath House P.O. Mezhathur Palakkad Dist.	04.09.1991	Adenocarcinoma
14.	Cattle Breeding Farm, Thumboormuzhi	10.09.1991	Adenocarcinoma
15.	Mr. Kuttappan Anappara, Manhapra Ernakulam Dist.	30.09.1991	Transitional cell carcinoma
16.	Mrs. Rita Vazhayal Lakh Colony Kallekulan, Poonjar Kottayam District	24.10.1991	Undifferentiated carcinoma
17.	Mr. M.P. Francis Kuttikkath House P.O. Cheranellur	26.10.1991	Adenocarcinoma
*18.	Smt. Mariamma Varkey Paloor House Puthuppally	01.11.1991	Undifferentiated carcinoma
*19.	Mr. Jaison Abraham Kallorkactil Moovatupuzha	09.11.1991	Adenocarcinoma
*20.	Mr. Ramanujan Mullakkara House Wadakkanchery	09.11.1991	Transitional cell carcinoma
*21.	Mr. Muralidharan P.O. Poothole Thrissur Dist.	15.11.1991	Papillary adenocarcinoma
22.	Mr. Ganapathy S/o Kunhayyappan Padiyam Village Thrissur Dist.	12.12.1991	Adenocarcinoma
23.	Vazhangat Sarojini W/o Vasudevan Thenhippalam	22.12.1991	Squamous cell carcinoma

* Animals died

Table 10. AgNOR count in tumour cells of ethmoid carcinoma

Sl. No.	Histopathology	Slide (a)	Slide (b)	Slide (c)	Slide (d)	Average
1.	Adenocarcinoma	2.38 ± 0.08	1.17 ± 0.10	1.47 ± 0.06	1.73 ± 0.08	1.69 ± 0.26
2.	"	1.44 ± 0.06	1.54 ± 0.06	2.01 ± 0.09	1.65 ± 0.07	1.66 ± 0.12
3.	"	2.20 ± 0.08	1.67 ± 0.08	1.16 ± 0.04	1.07 ± 0.03	1.52 ± 0.26
4.	"	1.40 ± 0.05	1.05 ± 0.02	1.26 ± 0.05	1.27 ± 0.05	1.24 ± 0.07
5.	"	2.52 ± 0.09	1.74 ± 0.08	1.83 ± 0.06	2.1 ± 0.07	2.04 ± 0.17
6.	Squamouscell carcinoma	2.69 ± 0.11	1.24 ± 0.05	1.71 ± 0.09	1.60 ± 0.07	1.81 ± 0.31
7.	"	2.73 ± 0.11	1.84 ± 0.08	2.17 ± 0.09	2.43 ± 0.10	2.29 ± 0.19
8.	Adenocarcinoma	1.97 ± 0.06	1.75 ± 0.07	1.77 ± 0.06	1.13 ± 0.06	1.78 ± 0.07
9.	"	2.23 ± 0.05	1.76 ± 0.06	1.95 ± 0.06	1.78 ± 0.08	1.93 ± 0.108
10.	"	1.41 ± 0.05	1.79 ± 0.06	1.08 ± 0.06	1.70 ± 0.06	1.65 ± 0.08
11.	"	1.61 ± 0.06	2.02 ± 0.08	1.81 ± 0.06	1.89 ± 0.08	1.83 ± 0.08
12	"	1.96 ± 0.09	2.26 ± 0.08	2.30 ± 0.07	2.38 ± 0.08	2.22 ± 0.09
13.	"	1.97 ± 0.06	1.87 ± 0.06	2.22 ± 0.07	1.96 ± 0.07	2.00 ± 0.07
14.	"	2.20 ± 0.07	1.98 ± 0.06	1.82 ± 0.06	1.90 ± 0.05	1.97 ± 0.08

I Adenocarcinoma 1.79 = 1.79 ± 0.08
n = 12 SE = 0.08

II Squamous cell carcinoma 2.05 = 2.05 ± 0.24
n = 2 SE = 0.24

Table 11. Serum zinc levels in tumour bearing animals and non tumour bearing animals

Tumour bearing animals		Non tumour bearing animals	
1.	0.84 ppm	1.	2.52 ppm
2.	1.74 ppm	2.	2.10 ppm
3.	1.14 ppm	3.	0.66 ppm
4.	0.66 ppm	4.	2.64 ppm
5.	1.50 ppm	5.	1.02 ppm
6.	1.32 ppm	6.	0.66 ppm
7.	1.44 ppm	7.	1.80 ppm
8.	1.08 ppm	8.	0.84 ppm
9.	0.66 ppm	9.	0.72 ppm
10.	0.84 ppm	10.	0.60 ppm
11.	0.60 ppm	11.	0.60 ppm
12.	0.66 ppm	12.	2.04 ppm
13.	0.48 ppm		
14.	2.04 ppm	Mean = 1.35	SE = 0.2322029
15.	0.24 ppm	= 1.35 ± 0.23 ppm	
16.	1.44 ppm		
17.	0.36 ppm		
18.	0.90 ppm		
19.	0.84 ppm		
Mean = 0.9884209 SE = 0.1113902			
= 0.99 ± 0.11 ppm			

Table 12. Serum copper level in tumour bearing animals and non tumour bearing animals

Tumour bearing animals		Non tumour bearing animals	
1.	0.48 ppm	1.	1.44 ppm
2.	1.18 ppm	2.	1.22 ppm
3.	1.04 ppm	3.	1.54 ppm
4.	0.92 ppm	4.	1.00 ppm
5.	0.92 ppm	5.	1.12 ppm
6.	1.36 ppm	6.	0.94 ppm
7.	0.88 ppm	7.	0.78 ppm
8.	0.96 ppm	8.	0.88 ppm
9.	0.70 ppm	9.	0.88 ppm
10.	0.94 ppm	10.	0.78 ppm
11.	0.84 ppm	11.	0.78 ppm
12.	0.92 ppm	12.	1.10 ppm
13.	1.04 ppm		
14.	0.68 ppm	Mean =	1.04 SE = 0.073
15.	0.78 ppm	=	1.04 $\pm$ 0.07
16.	0.98 ppm		
17.	0.40 ppm		
18.	0.26 ppm		
Mean =	0.857894 ppm SE = 0.0608		
=	0.86 $\pm$ 0.06 ppm		

Table 13. Parentage of ethmoid carcinoma animals and their sire and dam

Sl.No.	Animal number	Date of birth	Breed	Sire	Dam
Ethmoid carcinoma animal					
1.	005	10.04.1984	CB	JF 23	432
2.	017	21.09.1979	CB	J 204	5121
3.	068	09.02.1980	CB	369	625
4.	096	03.05.1980	CB	1028	A 685
5.	510	11.06.1982	CB	KJ 25	24159
6.	700	06.04.1983	CB	KJ 25	10862
Sire of ethmoid carcinoma animal					
1.	JF 23	--	Holstein	--	--
2.	J 204	--	--	--	--
3.	369	--	--	--	--
4.	1028	28.08.1975	Karan Swiss	BS 437	BC 531
5.	KJ 25	18.07.1978	Kankrej Jersey cross	--	--
Dam of ethmoid carcinoma animal					
1.	432	--	--	--	--
2.	5121	--	--	--	--
3.	625	08.04.1974	--	052	645
4.	A 685	29.04.1977	--	--	432
5.	24159	18.02.1972	--	Rodel	ND
6.	10862	01.11.1972	--	Vedy	ND

Discussion

DISCUSSION

Ethmoid carcinoma in domestic animals is an important emerging neoplastic condition in Kerala. This was encountered in Kerala Since 1960. During the 13 year period from 1960 to 1972, 92 cases were observed and during 1977 to 1979 period 150 cases were recorded from Kerala (Rajan, 1980). During the present period of study, 1989 to 1991, 285 cases of ethmoid carcinoma were recorded from the state of Kerala. This is a reflection of the magnitude of increase of incidence of the tumour. It may also be pointed out that these are only the cases which were brought to the notice of the department and the Veterinary Officers. There may be ever so many cases which are not reported. The true incidence will perforce to be very high. This observation points out the importance of this tumour.

Sreekumaran and Rajan (1983) observed that this tumour has established itself in an endemic form in Kerala and reported that all districts of the state are equally affected. District-wise distribution of the ethmoid carcinoma cases, based on the reports received during the present study showed that reported cases were highest in Thrissur district (21.57 per cent) and recorded cases from the Kasaragod district was the lowest (0.35 per cent).

This difference in the report of the incidence of the condition is related to the distance between this institution and various districts and the density of population of cattle in various districts.

During the present study highest percentage of report was during March (12.92 per cent) and lowest was in June and September (5.78 per cent), though there was report of this condition throughout the year. This higher percentage of report of the condition in March may be related to the high atmospheric temperature during that month. Cross-bred animals are less tolerant to high atmospheric temperature and they dessipate the high body temperature by increasing the respiratory rate. As the main clinical symptom of this condition is respiratory distress due to obstruction in the nasal passage by the tumour mass, the animals affected by this condition may strain too much out of heat stress and the blockage in the respiratory passage. This aggravated clinical symptoms are noticed by the owners and cases are identified. This will explain the higher percentage of report of this condition during March.

Rajan (1980) stated that there was relatively high reporting of incidence during the months of July and August. He observed that, this period of the year being rainy season, animals will be stall fed, hence there will be close observation by the owners, and cases will be identified early and this be

considered as the reason for higher percentage of report during this period.

In India, the earlier reports on the incidence of this condition was from Southern states like Andhra Pradesh (Narayana, 1960 and Sastry and Rao, 1964), Kerala (Rajan et al. 1972), Tamil Nadu (Damodaran et al. 1974), Karnataka (Balasubrahmaniyam,1975) and Orissa (Nayak et al. 1979, 1980). Recent published reports indicates that this condition has spread to other Northern states of India (Swarup et al. 1987; Chakraborti et al. 1988). These observations would, therefore, imply that incidence of this condition is on the increase in Southern states of India, and it has spread to other parts of the country, and this subcontinent has become an endemic zone of ethmoid carcinoma in domestic animals.

It is of significance to observe that ethmoidal neoplasm which was frequent in Scandinavian countries at the beginning of the century, has in recent times become practically a non-existant entity in these countries. While in South India the incidence of the tumour which was sporadic in the beginning, appears to be on the increase in recent years.

Twenty-three cases studied were histologically classified as adenocarcinoma (14), papillary adenocarcinoma (2), squamous cell carcinoma (3), transitional cell carcinoma (2), and undifferentiated carcinoma (2). The gross and histological

studies have clearly indicated that the tumour is a primary growth arising from the mucosa of the ethmoid. Inada and Tokarnia (1973) indicated that the histogenesis of the tumour is from the Bowman's gland of the ethmoid mucosa. The presence of transitional stages in adenocarcinoma would suggest that the tumours are primarily adenocarcinoma and squamous cell carcinomas are the manifestation of metaplasia of columnar epithelial cells. The common histological types of tumour was adenocarcinoma. In many of the squamous cell carcinomas there were areas of transition from columnar to squamous type. Both keratinising and non-keratinising squamous cell carcinomas were encountered. The undifferentiated carcinomas consisted of sheets of proliferating epithelial cells. Magnusson (1916) opined that adenocarcinoma originated from the glands of ethmoid mucosa and squamous cell carcinoma from the surface epithelium of the ethmoidal mucosa which had undergone squamous metaplasia. Histological types observed by earlier workers were adenocarcinoma, squamous cell carcinoma and undifferentiated carcinoma (Stenstrom, 1915; Nair and Sastry, 1954; Rajan et al. 1972; Nair, 1973; Damodaran et al. 1974; Balasubrahmaniyam, 1975; Jayaraman et al. 1979; Sreekumaran, 1980; Rajan, 1980; Rajan and Sulochana, 1982; Choudhary and Rao, 1982; Sreekumaran and Rajan, 1983; Chakraborti et al. 1988 and Murali Manohar, 1988). Nair (1973) and Balasubrahmaniyam (1975) also recorded transitional cell carcinoma along with the other three types. The histological types of tumour encountered in the present study were similar to those reported by earlier workers.

Ethmoid carcinoma in domestic animals is an important neoplastic condition in Kerala. The etiological aspects of this tumour is not fully understood. Knowledge on the biological behaviour of the neoplastic cell is still incomplete. In the present study attempts were made to grow the tumour cells in vitro. Tumour tissue was collected from nineteen cases for cell culture study. Medium 199 (Hi Media), DMEM (Hi Media) and RPMI- 1640 (Hi Media) with either ten per cent foetal calf serum or ten per cent goat serum were the different media employed in this study to support the growth of tumour cells in artificial cell culture system. In spite of taking all precautions to avoid contamination in cell culture and employing meticulous aseptic techniques, it was not possible to grow the tumour cells in culture media except in one case. In one case RPMI - 1640 (Hi Media) with ten per cent foetal calf serum was found to support the growth. Histologically the tumour was diagnosed as adenocarcinoma.

The cells in culture showed active proliferation and monolayer formation. Monolayer became confluent within 13 days and first passage could be carried out successfully. In culture, the cells were well spread with epithelial type morphology, predominating cell type being columnar. The smear prepared from the cell suspension for seeding in first passage also showed large cells with well defined nucleus and distinct cytoplasm. The nucleus was oval or round. Cells in various

stagesof division were also seen in the smear. Thus it appears to be the first report of successful cultivation of ethmoid carcinoma cells from cattle in tissue culture media. This success could be achieved only in the eighteenth attempt. Reduced viable cell count, and primary contamination from the tumour mass were the main problems in other attempts. Heavy antibiotic and antifungal agent supplementation could control the contamination in five cases, but in such occasions, the cells were neither adhering to the glass surface nor dividing in the suspension. Increased amount of dead cells in the tumour cell suspension prepared for seeding was perhaps due to the advanced necrobiotic changes in the tumour mass.

The explant culture as such was a failure due to the inherent bacterial and fungal contamination, in the tumour explant itself, which could not be controlled even after the addition of high doses of antibiotics and antifungal agents. Various workers had attempted to culture the bovine ethmoid carcinoma cells in artificial media without success (Jayaraman et al. 1979; Sulochana, 1980; Pospischil et al. 1982 and Karki and Rajan, 1986). Sulochana (1980) attributed the failure of the growth of the tumour cells in culture media to the absence of certain unknown factors required by the tumour cell for growth or possibly due to the presence of some infectious agent in the tumour cell itself. She employed HBSS with 0.5 per cent lactalbumin hydrolysate, 0.15 per cent yeast extract. 200 IU of

penicillin, 200 micrograms of streptomycin, 40 micrograms of kanamycin, 10 micrograms of gentamycin and ten per cent calf serum as the growth media. Karki and Rajan (1986) opined the possible role of endogenous infectious agent, degenerated condition of the tumour tissue and absence of certain unknown factors required by the neoplastic cells for growth as the factor responsible for failure.

They employed HBSS, TC 199 (Difco) and DMEM (Difco) supplemented with 10 to 20 per cent calf serum as the growth medium. As the tumour is deep seated, in the paranasal sinuses it has access to the atmospheric air that is inhaled and exhaled, therefore the tumour tissue often get heavily contaminated with microbes. Active respiration by the ailing animals due to the obstruction in the nasal passage, makes the tumour tissue more predisposed for contamination. In field conditions, the disease is diagnosed at a late stage and by this time, the tumour mass would have already undergone necrotic changes, reducing the viable cells in the tumour tissue available for culture. The possibility of the abundant dead cells, releasing certain toxic factors, which will inhibit the growth of viable cells if any cannot also be ruled out. To contain the heavy contamination, higher doses of antibiotics were added in the tissue culture media, this might also inhibit the growth of neoplastic cells.

The success rate could be improved, if the tumour tissue is available in the fresh condition without necrosis and

contamination. The earlier workers had attributed the failure of growth to the absence of certain unknown factors in the growth media as one of the reasons. In the present study, RPMI-1640 with 10 per cent foetal calf serum was found to support the growth of tumour cells in the artificial media. The failure to support the growth of tumour cells by Medium - 199 (Hi Media) and DMEM (Hi Media) is due to the absence of certain factors and variation in the concentration of certain nutrients in the media. A change in the source of serum from foetal calf to goat also did not promote the growth. From the present study, it is clear that RPMI - 1640 (Hi Media) contained that unknown factors which were not present in the media used by earlier workers like HBSS, TC-199 (Difco) and DMEM (Difco) and in the present study Medium - 199 (Hi Media) and DMEM (Hi Media). RPMI - 1640 (Hi Media) differs from Medium - 199 (Hi Media) and DMEM (Hi Media) in the following aspects. The percentage of calcium and magnesium in RPMI - 1640 is less compared to the other media employed. The source of Ca in RPMI-1640 is calcium nitrate 4 H_2O instead of calcium chloride 2 H_2O in other media. The components in RPMI-1640 which showed higher concentration compared to other Media employed were Arginine, Cystine, Biotin, Myo-Inositol, P-amino benzoic acid, D-Glucose and Glutathione (reduced). The higher concentration of Arginine, Cystine, Biotin, Myo-Inositol, P-amino benzoic acid, D-Glucose and glutathione (reduced) and exclusive component of L-Asparginine and Vitamin B-12 may be the reason for RPMI-1640 promoting the growth of ethmoid carcinoma cells in cell culture system.

Gamma glutamyl transpeptidase is an enzyme involved in the drug detoxification mechanism. This enzyme is normally present in the kidney, liver, pancreas and intestine. Any pathological condition especially obstructive lesions in their ducts will elevate its level and it will be reflected in the serum GGT level also. Calderon (1985) demonstrated GGT activity in pre-cancerous lesions and carcinoma of oral, pharyngeal and laryngeal mucosa in human beings. Kalengayi et al. (1975) observed that expression of GGT was a common findings in liver lesions induced by aflatoxin B_1, a genotoxic carcinogen. Rao et al. (1988) demonstrated lack of expression of GGT mRNAs in liver tumour induced by peroxisome proliferators and non genotoxic carcinogens like ciprofibrate and nafenopine. Rajan et al. (1972) and Pospischil et al. (1979) reported simultaneous occurrence of ethmoid carcinoma in animals and mycotoxins in feed. They pointed out that the role of mycotoxins has to be assessed in causing the tumour, though the direct evidence for the involvement of toxin was lacking. However, in pigs, there was a direct correlation between the incidence of tumour and consumption of aflatoxin contaminated groundnut cake (Rajan et al. 1981). In the present study two sections each from twelve cases were employed for the histochemical demonstration of GGT. The tumour types included adenocarcinoma, papillary adenocarcinoma and squamous cell carcinoma. All the sections stained for GGT showed a positive reaction. However, normal ethmoid epithelium did not show the presence of GGT. There was

no difference in the expression of GGT in various histological types of tumour. The expression of GGT in histological sections can be considered as an indirect evidence for the involvement of a genotoxic carcinogen like aflatoxin, which is usually a contaminant in the cattle feed, in the causation of this tumour.

Zhang et al. (1981) had reported that, when groundnut cake was fed to breeding ows in 1973, signs of jaundice developed but disappeared on withdrawal of cake. However, in 1976, these animals died and post-mortem examination revealed tumourous growth in the ethmoidal sinuses and liver. Samples of mouldy groundnut cake was found to be heavily contaminated with spores of Aspergillus flavus. The aflatoxin B_1 content of the cake, assayed by thin layer chromatography was 250-301 ppb. This is a report on the spontaneous ethmoid tumour and liver tumours in pigs possibly due to consumption of aflatoxin B_1 contaminated feed. Larsson et al. (1989) examined whether the bovine nasal olfactory mucosa has a capacity to metabolise aflatoxin B_1 and found that the nasal olfactory mucosa has a much higher capacity than the liver to form lipid soluble, water soluble and tissue bound aflatoxin B_1 - metabolites. High resolution micro-autography showed a strong localisation of the tissue bound metabolites in the sustentacular cells in the apical portion of the olfactory surface epithelium and in Bowman's glands in the lamina propria mucosae. Especially in the sustentacular cells the labelling was preferentially located in the nuclei of the cells. They opined that, their study

strengthened the hypothesis that exposure of aflatoxin B_1 contaminated feed may be an important etiological factor in the development of nasal tumours in cattle. Larsson et al. (1990) also reported that experiments in vitro showed a capacity of the nasal glands to form tissue bound 3H aflatoxin B_1 metabolites in mice. To ascertain the role of aflatoxin B_1 in the causation of this tumour, long term experimental studies should be taken up by giving aflatoxin B_1 in a naturally susceptible host.

Sordhal et al. (1969) and Poderson et al. (1970) stated that mitochondria in tumour cells were defective functionally. Nair (1980) and Nair et al. (1987) studied the ultrastructure of the ethmoid carcinoma cells in detail. They found varied number of round or oval or slightly elongated mitochondria with prominent cristae. Transverse or ring shaped cristae were noticed in some mitochondria while in others, there was complete disorganisation and dissolution. Although in general mitochondria were randomly distributed, occasional close association between rough endoplasmic reticulam and mitochondria was seen.

In the present study special stains for the demonstration of mitochondria showed diffuse greenish colouration of the cytoplasm and some cells with granules of various sizes were noticed indicating pronounced mitochondrial proliferation and/or damage to the mitochondria. There was no difference in their expression in various histological types of tumour. The diffuse

greenish discolouration of the cytoplasm may be attributed to the damage of mitochondria and release of phospholipids into the cytoplasm, which will be stained by luxol fast blue or due to pronounced proliferation of mitochondria as observed in ultra-structural section by Nair et al. (1987). The histochemical demonstration of mitochondria in tumour cells indicated the presence of abnormal and degenerated mitochondria in the cytoplasm of neoplastic cells. This observation supports the ultrastructural demonstration of increased and abnormal mitochondria in tumour cells.

It was the province of cytogeneticist to locate the AgNORs in the metaphase chromosome spreads. They are the chromosomal segments in which ribosomal RNA (rRNA) is encoded and they are thus responsible for the development of RNA containing nucleolus or nucleoli into which the NORs project on large loops of DNA. The silver staining does not identify neither rRNA nor rDNA, but it demonstrates the acidic proteins associated with these sites of rRNA transcription. Ploton et al. (1986) improved the staining technique and visualisation of argyrophilic proteins of the AgNORs at optical level and observed that NOR number appeared to reflect cell and nuclear activity. Crocker and Nar (1987) observed that there was significant difference between the number of AgNORs in low grade lymphoma and high grade limphoma. Denham and Solisbury (1988) studied AgNORs in kerato acanthoma and squamous cell carcinoma. They observed AgNOR count of 2.5 in the range of 1.4 to 2.6 in

kerato acanthoma and 2.1 in the range of 1.4 to 3.2 in squamous cell carcinoma. McNicol et al. (1988) studied AgNORs in pitutary adenomas, and it varied from 1.69 ± 0.11 to 2.18 ± 0.15. Boon and Sharif (1988) evaluated the prognostic value of the AgNOR technique in Meningioma and opined that, this technique was not useful for predicting the biological behaviour of meningiomas. Coombe et al. (1988) recorded a count of 2.12 - 2.37 in ovarian adenocarcinomas in human beings. Underwood and Giri (1988) suggested that the higher AgNOR count reported in certain tumours represented not so much an absolute increase in the numbers of AgNORs but were attributable rather to increased transcriptional activity and nucleolar dispersion both of which make it easier to identify and count individual AgNORs. Crocker and Egan (1988) observed that in low grade Non-Hodgkins lymphoma, the AgNOR sites were significantly larger than in high grade Non-Hodgkins lymphoma. Howat et al. (1988) in their study concluded that AgNOR counting technique was of no value in predicting prognosis of cutaneous malignant melanoma. Giri et al. (1989) concluded that AgNOR method alone did not offer a reliable histological determinant for malignancy in the breast. Griffith et al. (1989) concluded that, assessment of NOR activity was not reliable in routinely formalin fixed archival tissues. Tidsley et al. (1990) could not find any significant correlation between AgNORs and tumour grade or stage in colorectal carcinoma.

From the above reports it appears that AgNOR counting is not reliable in making a prognosis or histological differentiation or staging in epithelial tumours, meningiomas, ovarian adenocarcinomas, cutaneous malignant melanomas, malignancy of the breasts and colorectal carcinomas. But it appears as a reliable technique in staging lymphomas, as low grade or high grade. In the present study the average count for AgNOR in adenocarcinoma was 1.79 ± 0.08 in the range of 1.24 ± 0.07 to 2.22 ± 0.09 and in squamous cell carcinoma, the count was 2.05 ± 0.24 in the range of 1.81 ± 0.31 to 2.29 ± 0.19. Here there was a slight increase in the AgNOR count in squamous cell carcinoma compared to adenocarcinoma, but this was not statistically significant. This difference between AgNOR counts in the two histological types of tumour does not reflect much on their differentiation or cellularity. The count of AgNOR in these cases was as low as in the case of cutaneous tumours like kerato acanthoma, and squamous cell carcinoma and colorectal carcinoma. It can, therefore be considered that, the AgNOR counting as a marker is of no importance in ethmoid carcinoma to differentiate histological types.

Literature on the microelement status in animal cancer patients is scanty. Fischer et al. (1973) and Miles (1985) opined that decreased level of serum zinc is associated with carcinogenesis. Serum copper level was shown to be a non-specific but reliable indicator in Hodgkins disease (Reddy et al. 1980). Varghese et al. (1987) reported a significant

reduction in serum copper and zinc level in oral cancer patients of Kerala, and even in the pre-cancerous lesions like oral submucous fibrosis (OSMF). In the present study also serum copper and zinc levels were low as compared to the healthy controls, but it was not statistically significant. The reduction in serum copper and zinc levels may be due to general reduction in the condition of the animals due to reduced feed consumption and cachexia. This aspect requires further detailed study.

The incidence of this tumour is on the increase in Kerala in recent years. Due to intensive cattle cross breeding programme followed in the state, the population of cross-bred animals also is on the increase. The reports of sinus tumour animals are mainly from cross-bred animals. There is increased incidence of this condition in the University Livestock Farm, Mannuthy, were all the animals were cross-bred and even there is possibility of inbreeding. This intensive cross-breeding may induce alterations in the genetic make up of animals, which make them predisposed to ethmoid carcinoma. During the present period of study, six cases were reported from the University Livestock Farm, Mannuthy. On examination of the parentage of these animals, there was a common sire for two sinus tumour animals. These data are not sufficient to make any conclusion on the genetic predisposition of this condition. This may be quite incidental. Detailed karyological studies should be taken

up to find out genetic predisposition, if any. Jayaraman et al. (1979) also reported that, there was preponderance of the incidence in the progeny of few sires indicating genetic predisposition. Kornel et al. (1984) reported high incidence of ethmoturbinate neoplasm in a pure-breed Jersey herd. They observed that, two bulls of their farm were the potent source of transmission as their daughters suffered from ethmoturbinate neoplasm ranging from 80 to 100 per cent. Not only their daughters, but the grand daughters also died due to this neoplasm. It was considered that there is a possible vertical transmission of ethmoid neoplasm among cows.

Summary

SUMMARY

1. During the period of study (1989 to 1991) 519 nasal smears from cattle suspected to have ethmoid carcinoma were received for exfoliative cytology study and 285 were found positive for ethmoid carcinoma.

2. Month-wise analysis of incidence of this condition revealed that higher percentage of incidence was in March (12.92 per cent) and lowest was during June and September (5.78 per cent).

3. District-wise distribution of ethmoid carcinoma cases was studied. Highest percentage of reported incidence (21.57 per cent) was from Thrissur district. Kasaragod district had the lowest percentage of report (0.35 per cent).

4. During the period of study, 23 tumour bearing animals were procured. They belonged to University Livestock Farm, Mannuthy (6), Cattle Breeding Farm, Thumboormuzhi (1), and Private owners in and around Thrissur and neighbouring districts like Palakkad, Malappuram, Ernakulam and Kottayam (16).

5. Histopathologically, the tumours encountered were classified adenocarcinoma (14), papillary adenocarcinoma (2), squamous cell carcinoma (3), transitional cell carcinoma (2) and undifferentiated carcinoma (2).

6. Tumour tissue was collected from 19 animals for cell culture study. Explantation technique and cell dissociation technique were employed. RPMI-1640 (Hi Media), Medium-199 (Hi Media) and DMEM (Hi Media) with 10 per cent foetal calf serum or 10 per cent goat serum were the growth media used. Explantation technique was not useful in establishing the growth of the tissue. In cell dissociation technique with trypsin, RPMI-1640 with 10 per cent FCS was found to support the growth of ethmoid carcinoma cells in one instance.

7. There was expression of GGT in tumour tissue. There was no variation in the expression of the enzyme activity in various histological types. The expression of GGT indicated the possible aetiologic role of genotoxic carcinogen like aflatoxin.

8. Mitochondria were histochemically demonstrated in ethmoid carcinoma sections using luxol fast blue. Diffuse greenish colouration of the cytoplasm was observed indicating mitochondrial damage or pronounced proliferation. Twelve cases were studied, which included one case of papillary adenocarcinoma, nine cases of adenocarcinoma and two cases of squamous cell carcinoma. There was no qualitative and quantitative difference in the distribution of mitochondria in various histological types.

9. AgNOR count was made in ethmoid carcinoma sections using argyrophilic technique. They included 2 cases of adenocarcinoma and 12 cases of squamous cell carcinoma. Adenocarcinoma cases showed an average count of 1.79 ± 0.08, within the range of 1.24 ± 0.07 to 2.22 ± 0.09. Squamous cell carcinoma cases had an average count of 2.05 ± 0.24, within the range of 1.81 ± 0.31 to 2.29 ± 0.19. The difference was not statistically significant.

10. Serum zinc and copper level of 19 tumour bearing animals and 12 healthy cows were estimated employing atomic absorption spectrophotometer. Average serum zinc level in normal animals was 1.35 ± 0.23 ppm and copper level was 1.04 ± 0.07 ppm. The level of serum zinc in tumour animals was 0.99 ± 0.11 ppm and serum copper level was 0.86 ± 0.06 ppm. There was reduction in both serum zinc and copper level in the tumour bearing animals when compared to the non-tumour bearing healthy animals. However, the difference was not statistically significant.

11. Parentage of six ethmoid carcinoma bearing animals were examined. It revealed that two of the sinus tumour animals had a common sire. The need for further studies was indicated.

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AN ASSESSMENT OF THE BIOLOGICAL CHARACTERISTICS OF THE NEOPLASTIC CELLS OF ETHMOID CARCINOMA IN CATTLE

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ABSTRACT OF A THESIS

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ABSTRACT

Ethmoid carcinoma is an important neoplastic condition of domestic animals. Two hundred and eighty five positive cases were reported to the Centre of Excellence in Pathology, during 1989 to 1991. The incidence was reported throughout the years though highest was during March (12.92 per cent) and lowest was in June and September (5.78 per cent). The report of the incidence was more from Thrissur district (21.57 per cent) compared to other districts of Kerala. Twenty-three animals were procured from various farms of the University (7) and private owners, in and around Thrissur, and neighbouring districts, like Malappuram, Palakkad, Ernakulam and Kottayam (16). Histopathologically the tumours were diagnosed as Papillary adenocarcinoma (2), Adenocarcinoma (14), Squamous cell carcinoma (3), Transitional cell carcinoma (2) and Undifferentiated carcinoma (2). Efforts were made to culture ethmoid carcinoma cells *in vitro*, using various tissue culture media. However, the efforts were not successful in most cases. In one case RPMI-1640 (Hi Media) with 10 per cent foetal calf serum was found to support the growth of ethmoid carcinoma cells *in vitro*.

Histochemically GGT was demonstrated in tissue sections. There was no difference in the expression pattern of GGT in different histological types like adenocarcinoma and squamous cell carcinoma. This was considered as an indirect evidence for

the involvement of a genotoxic carcinogen like aflatoxin in the causation of this tumour. Special staining with luxol fast blue to demonstrate mitochondria was carried out in tumour sections. Diffuse greenish colouration of the cytoplasm of the neoplastic cells was observed indicating pronounced mitochondrial proliferation and/or damage of the mitochondria irrespective of the histological types like adenocarcinoma and squamous cell carcinoma. AgNOR count in ethmoid carcinoma was evaluated. An average count of 1.79 ± 0.08 was observed in adenocarcinoma cases, whereas squamous cell carcinoma cases showed an average AgNOR count of 2.05 ± 0.24. This difference was not statistically significant. Serum zinc level and copper level of sinus tumour animals was compared with that of normal animals. Tumour animals had a serum zinc level of 0.99 ± 0.11 ppm, and serum copper levels was 0.86 ± 0.06 ppm. The serum zinc level in normal animals was 1.35 ± 0.23 ppm and serum copper level was 1.04 ± 0.07 ppm. Though, there was a reduction in serum zinc and copper levels in tumour animals compared to normal animals, it was not statistically significant. Parentage of six ethmoid carcinoma animals of University Livestock Farm, Mannuthy was examined. Two ethmoid carcinoma animals of the University Livestock Farm, Mannuthy had a common sire. This aspect requires further study to clarify the genetic predisposition to this condition, if any.