Characterization of early cytogenetic changes and evaluation of the role of bile acids in the pathogenesis of Barrett's oesophagus.

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Characterization Of Early Cytogenetic Changes And Evaluation Of The Role Of Bile Acids In The Pathogenesis Of Barrett's Oesophagus.

by

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SUMMARY

Barrett's oesophagus is a pre-malignant condition, the incidence of which is steadily increasing. The exact cause of this condition is not known although it is strongly associated with reflux of gastric and duodenal contents. The early part of this study deals with the development of an in-vitro model of Barrett's cell culture to better understand the mechanism involved in neoplastic progression. Despite limited success our study shows that long term culture of Barrett's cells is extremely difficult, labor intensive and not easily reproducible. Cytogenetic evaluation of cultured cells using Fluorescent In-situ Hybridisation showed that amplification of chromosome four and eight in early stages of Barrett's. It is difficult to draw a conclusion from this study owing to the small number of samples analyzed. The latter part of the study deals with the evaluation of cytotoxic and genotoxic effect of bile acids on oesophageal cell lines using the cytokinesis-blocked micronucleus assay, a multi end point assay. Bile has been increasingly implicated in the pathogenesis of Barrett's although evidence regarding genotoxic and cytotoxic effect of various bile acids on oeophageal cell lines is limited. Our study clearly shows that all bile acids induce a significant cytotoxicity at concentrations which are significantly relevant in patients with Barrett's. However all bile acids except DCA lacked significant genotoxic effect in-vitro. Bile salts and acid together had a synergistic effect on cell toxicity. DCA induced significant DNA damage and apoptosis at concentrations as low as 100-200 μmol/liter. Further evaluation of the mechanism of DNA damage by DCA showed that it was predominantly a clastogen in nature. This study provides more evidence to show that DCA can cause DNA damage and apoptosis in oesophageal cell lines. We therefore conclude that un-conjugated bile acids (DCA) at neutral pH could

play an important role in the development of Barrett's metaplasia and its progression to oesophageal adneocarcinoma.

ABBREVIATIONS

ABL gene	Abelson proto-oncogene
ADCC	Adenocarcinoma of the Cardia
AGF-sup	Autologus Growth Factors / Cytokines
ALA	5-amonolevulinic acid
AP-1	Activator Protein 1
APC	Adenomatous Polyposis Coli Protein
ATF	Activated transcription factor
ATPase	Adenine Triphosphatase
ATM	Ataxia Telangiectasia Mutated
BA	Bile Acids
BCR	Breakpoint Cluster Region
BE	Barrett's Oesophagus
BN	Binucleate
BRI	Bile Reflux Index
BSA	Bovine Serum Albumin
BSG	British Society of Gastroenterologist
CBMN	Cytokinesis Blocked MN Assay
CDK	Cyclin-Dependent Kinase
CGH	Comparative Genomic Hybridaztion
CHO	Chinese Hamster Ovary
CKI's	CDK Inhibitor Protein
CMO	Chief Medical Officer
CO2	Carbon Dioxide
COM	Committee on Mutagenicity of Chemicals In Food, Consumer Products And The Environment
CREST	Calcinosis, Raynaud phenomenon, Oesophageal dismotility, Sclerodactyl, and telangiectasia
DAG	Diacyl Glycerol
DAPI	4', 6-diamidine-2'-phenylindole dihydrochloride
DCA	Deoxycholic acid
DEN	Diethyl Nitrosamine
DGER	Duodenal-Gastro-Oesophageal Reflux
DMEM	Dulbecco's Modified Essential Medium
2,6-DMNM	2,6-Dimethyl Nitrosomorphine
DMSO	Dimethyl Sulfoxyde
DNA	Deoxy-Ribonucleic Acid
DP Protein	DP Dimerization Protein
EACC	European Collection Of Cell Cultures
EAC	Oesophageal Adenocarcinoma
EBV	Epstien Barr Virus
EDTA	Ethylenediamine Tetra-acetic Acid
E2F	Factor Interacting With Adenovirus E2-Promoter

EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK's	Extra-Cellular Signal Regulated Kinases
FBS	fetal bovine serum
FCS	Fetal Calf Serum
FISH	fluorescence in-situ hybridization
FITC	Fluorescein Isothiocynate
GADD45	Growth arrest and DNA damage 45
GCA	Glycocholic acid
GDCA	Glycodeoxicholic acid
GERD	Gastro-Oesophageal Reflux Disease
GORD	Gastro Oesophageal Reflux Disease
HCl	Hydrochloride acid
HGD	High Grade Dysplasia
HIPEC	Lines Human Intestinal Primary Culture Lines
HpD	Hematoporphyrin Derivitive
HGPRT	Hypoxanthine Guanine phosphoribosyl transferase assay
HIFCS	Heat Inactivated Foetal Calf Serum
H.pylori	Helecobacter Pylori
ICPEMC	International Commission for Protection against Environmental Mutagens and Carcinogens
ICH	International Conferences on Harmonisation
IEC lines	Intestinal Epithelial Cells
IGF	Insulin-Like Growth Factor
INK4	Inhibitor of CDK4 & CDK6
IP3	Inositol 1-4-5-Triphosphate
LCA	Lithocholic Acid
LGD	Low Grade Dysplasia
LOH	Loss of Heterozygosity
MAK	Mitogen Activated Protein
MDM2	Murine Double Minute Clone 2
MFISH	Multiplex Fluorescence In-situ Hybridization
MLA	Mouse lymphoma assay
MMC	Mitomycin C
MN	Micronucleus
MOLY	Mouse Lymphoma LS1784
m-RNA	Messenger RNA
MTOC	Microtubule Organizing Centres
NaOH	Sodium Chloride
NSAIDS	Non Steroidal Anti-Inflammatory Drugs
OADC	Oesophageal Adenocarcinoma
OECD	The organisation For Economic Co-operation and Development Pharmaceuticals for Human Use
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PDT	Photodynamic Therapy

PIP2	Phosphatidylinosito 1-4-5-Disphosphate
PKC	Protein Kinase C
PLA2	Phospholipase-A2
PLC	Phospholipase C
PN	Polynuclear Cells
PpIX	Protoporphyrin IX
PPI	Proton Pump Inhibitors
PRb	Retinoblastoma Protein
RET	Rearranged during transfection
RGS	Reflux Gastritis Score
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
SCC	Squamous Cell Carcinoma
SIM	Specialised Intestinal Metaplasia
SKY	Spectral Karyotyping
SSC	Standard saline citrate
TCA	Taurocholic Acid
TCM	Transport Medium
TDCA	Taurodeoxycholic Acid
TFT	Trifluorothymidine
TGF's	Transforming Growth Factors
TGF-β1	Transforming Growth Factor Beta 1
TK	Thymidine Kinase
TNF's	Tumor Necrosis Factors
TXA2	Thromboxane A2

CHAPTER ONE

1.1 INTRODUCTION

The incidence of adenocarcinoma of the oesophagus has been steadily increasing over the last three decades in the Western world (Blot *et al.,* 1991; Pera *et al.,* 1993). In fact the increase has been more rapid than that of any other malignant neoplasm (Haggitt 1994). Oesophageal adenocarcinoma (OADC) is a lethal malignancy and most patients present with advanced disease at the time of diagnosis. The prognosis is poor with a very low five year survival rate(Clark *et al.,* 1996; Spechler 1996). Though a few moderately strong risk factors have been identified, the exact reasons for the rising incidences are unknown (Kim *et al.,* 1997). Virtually all adenocarcinomas arise from pre-existing Barrett's metaplastic tissue, as does at least 40% of the adenocarcinoma of the gastro-oesophageal junction (Pera *et al.,* 1993; Clark *et al.,* 1994). The increasing incidence of oesophageal adenocarcinoma could be a reflection of an increasing incidence of its precursor lesion Barrett's oesophagus (known as BE) (Prach *et al.,* 1997; DeVault 2000).

1.2 BARRETT'S OESOPHAGUS

1.21 DEFINITION

Barrett's oesophagus is defined as a condition in which the normal stratified squamous epithelium of the distal oesophagus is replaced by metaplastic columnar epithelium containing goblet cells (Haggitt 1994).

1.22 HISTORY

The earliest description of columnar lined oesophagus was by Tileston, a pathologist, who reported several patients who had "peptic ulcer of the oesophagus. He noted the close resemblance of the mucous membrane about the ulcer to that normally found in the stomach (Tileston W 1906). A number of other investigators described a similar kind of lesion in the subsequent years. Most of these investigators, including Norman Barrett, who described this lesion in 1950, believed that the columnar lined organ was not oesophagus at all, but rather a tubular segment of the stomach that had been tethered within the chest by a congenitally short oesophagus (Barrett NR 1950). In 1953 Allison and Johnstone proved that this columnar lined intra-thoracic structure was indeed oesophagus and not stomach (Allison *et al.,* 1953). They suggested that the ulceration in columnar lined oesophagus should be called "**Barretts ulcers**". In the early 1970's there was considerable disagreement between investigators as to whether the columnar lined epithelium of Barrett's oesophagus was gastric or intestinal type. This confusion was clarified by the work published by Paull *et al* in 1976 (Paull *et al.,* 1976). These investigators found that three types of columnar epithelium could be present in Barrett's oesophagus.

- Specialised columnar epithelium also called specialised intestinal metaplasia (SIM)
- Gastric fundic type epithelium with oxyntic glands containing chief and parietal cells, and
- Junctional type epithelium comprised exclusively of mucus secreting cells.

The latter two epithelial types can be indistinguishable from those normally found in the gastric fundus and cardia. The specialised intestinal metaplasia is clearly abnormal and can progress to dysplasia.

1.23 PATHOGENESIS AND SIGNIFICANCE OF BARRETT'S OESOPHAGUS

The prevalence of BE is increasing and so are its sequel (Prach *et al.,* 1997). BE is found in 0.8-2% of patients undergoing upper endoscopy and in 6-15% of patients who undergo endoscopy for symptoms of gastroesophageal reflux. The prevalence in the general population is not precisely known but some studies suggest a prevalence of 5-9% in a general population aged more than 50 years (Cameron *et al.,* 1990; Hirota *et al.,* 1999). Based on prospective studies, the incidence of adenocarcinoma developing from Barrett's oesophagus is approximately 1 per 100 patient-years (Drewitz *et al.,* 1997). This represents a 30- to 125-fold increased risk over the general population. Thus, Barrett's oesophagus is a relatively common lesion that has significant malignant potential. Approximately 5%-10% of patients diagnosed with *Barrett's* oesophagus are thought to be at risk of developing oesophageal adenocarcinoma (Spechler *et al.,* 1984; Cameron *et al.,* 1985). The risk of malignancy is lower in short segment BE than in long segment Barrett's oesophagus (Rudolph *et al.,* 2000).

Barrett's Oesophagus develops as a result of chronic duodeno-gastro-oesophageal reflux (DGER). It is believed that the chronicity of symptoms is more important than their severity in predicting Barrett's oesophagus. Barrett's mucosa by itself is usually asymptomatic and in-fact the metaplastic oesophageal mucosa may be less pain sensitive than the native squamous mucosa (Iascone *et al.,* 1983; Loffeld 2001).

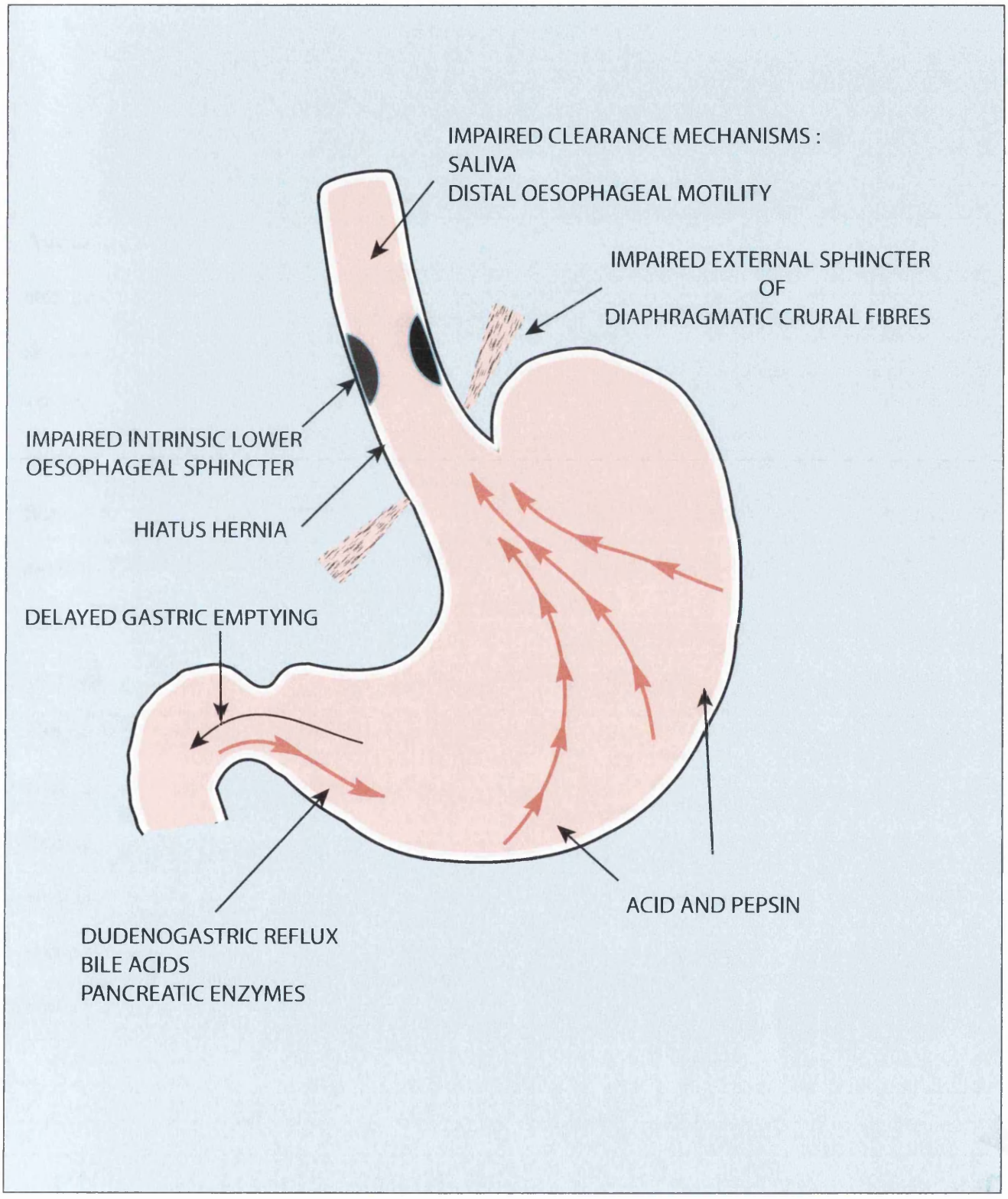
Although chronic DGER is the most significant risk factor, the exact role of continuous or intermittent acid reflux, bile or other duodenal contents still remains to be elucidated. Apart from gastroesophageal reflux, genetic factors appear to be involved in the development of Barrett's oesophagus as indicated by reports of high prevalence rates of BE in certain families (Everhart *et al.,* 1983; Crabb *et al.,* 1985) and by the fact that BE is rare among ethnic minorities (Smith *et al.,* 1984).

There are other risk factors which are of less significance like white race, male gender, obesity and affluent society. Helicobacter pylori infection (especially cagA gene positive strains) which is strongly associated with the development of intestinal metaplasia and cancer in the stomach (Stemmermann 1994; Asaka *et al.,* 1997) does not seem to have a positive association with BE or oesophageal adenocarcinoma (Talley *et al.,* 1988; O'Connor *et al.,* 1994; Abbas *et al.,* 1995; Liston *et al.,* 1996). In fact recent reports suggest that H.pylori infection may actually protect the oesophagus from cancer by preventing the development of reflux oesophagitis and Barrett's oesophagus (Labenz *et al.,* 1997; Werdmuller *et al.,* 1997; Weston AP 1998). It is proposed that H.pylori infection can cause pangastritis decreasing gastric secretion and hence preventing gastro-oesophageal reflux (Graham *et al.,* 1998) A number of physiological abnormalities predispose to Barrett's oesophagus (Spechler 2000). The proposed abnormalities are (see figure 1.1):

- Extreme lower oesophageal sphincter hypotension that predisposes to the reflux of gastric contents, which may be caustic due to high concentration of acid and refluxed bile
- Gastric acid hyper-secretion

FIGURE 1.1.
SCHEMATIC REPRESENTATION OF MECHANISMS LEADING TO GASTRO-OESOPHAGEAL REFLUX AND BARRETT'S OESOPHAGUS

Adapted from Spechler et al., 2000

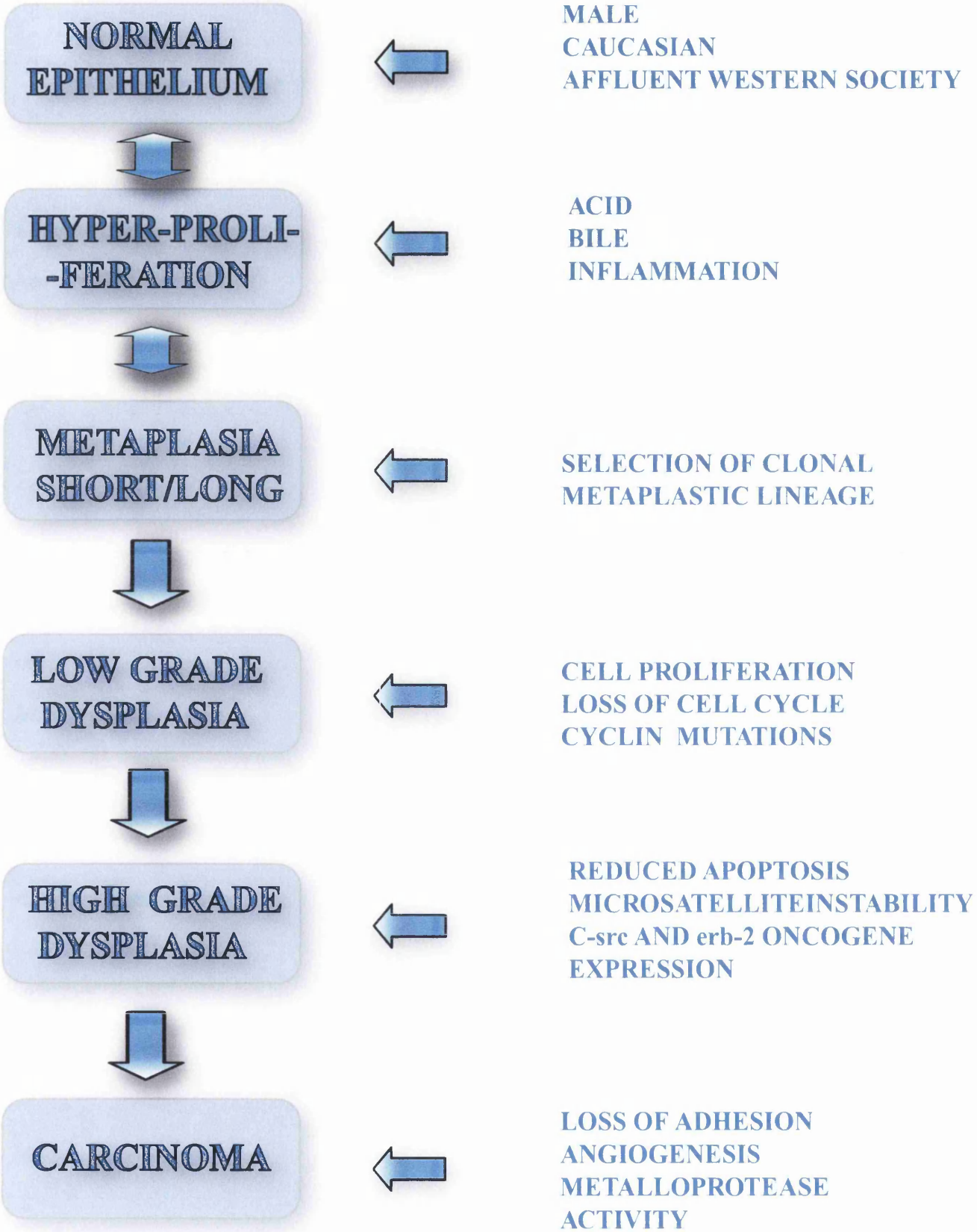


- Duodenogastric reflux
- Poor oesophageal contractility delays the clearance of the noxious refluxed material
- Diminished oesophageal pain sensitivity leads to loss of symptoms which otherwise would have warned the patient of oesophageal injury.
- Decreased secretion of epidermal growth factor in the saliva delays the healing of the reflux- damaged oesophagus.

Adenocarcinoma in BE does not arise de novo but appears to develop through a multi-step process with progressive worsening of a precursor lesion. This stepwise progression is recognised histologically as a metaplasia–dysplasia–carcinoma sequence (Reid *et al.,* 1992). The precursor lesion of adenocarcinoma is called dysplasia. Dysplasia can develop in short segment or long segment Barrett's but its prevalence is higher in long segment Barrett's (Weston *et al.,* 1997). Dysplasia is a morphological term, which etymologically means "malformation". It is derived from classic Greek and composed of 'dys' which means bad and 'plasis' which means form. It is defined as an unequivocal, non-invasive neoplastic transformation of the epithelium excluding all reactive changes.

The sequence of metaplasia-dysplasia–carcinoma is a multistep process and is associated with several changes in the gene structure, gene expression and protein structure (Jankowski *et al.,* 1999). Barrett's epithelium and its progression to adenocarcinoma is associated with a wide variety of cellular and molecular abnormalities. These include aneuploidy, chromosomal deletions and trisomy, microsatellite instability, single-gene alterations, in-appropriate production and response to growth factors, and alterations in

FIGURE 1.2 SCHEMATIC REPRESENTATION OF THE METAPLASIA DYSPLASIA CARCINOMA SEQUENCE (Adapted from Aldulaimi et al , 1999)



cellular adhesion molecules. These changes are mirrored by histologic progression through the metaplasia-dysplasia-carcinoma sequence (see figure 1.2).

The process by which squamous epithelium is replaced by specialized intestinal metaplasia is poorly understood. Prolonged reflux oesophagitis is associated with inflammation and cell death. Responses to the cell death and inflammation include an increase in the height of the zone of proliferation and length of basal papillae. This is accompanied by increased folding of the oesophageal epithelium, leading to stem cells at the tip of papillae becoming more superficial. These superficial stem cells may then be exposed to increased amounts of potentially noxious gastro-duodenal refluxate and oesophageal luminal contents. Such an environment may lead to stem cells dying or being damaged or selected if expressing the glandular phenotype of Barrett's epithelium (Jankowski *et al.,* 1999). Activation of specific oncogenes (especially epidermal growth factor receptor, c-erbB-2, H-ras and myc) and inhibition of specific tumour suppressor genes (especially p53 and p16) play an important role. One of the earliest events is thus the selection and propagation of the metaplastic clones with specialized intestinal metaplasia. Subsequently, altered expression of growth factors and genomic alterations of cell cycle-associated genes are noted.

1.231 ROLE OF ACIDS

Acid and pepsin play an important role in the oesophageal damage in patients with GERD. There is strong experimental and clinical evidence to support the role of acid and pepsin in the development of oesophageal injury. Studies have shown that reflux of acid with pepsin is more important in the induction of oesophageal damage than reflux of acid alone. It has been demonstrated that patients with GERD and BE have increased

frequency and duration of acid reflux (Hennessy 1985; Zamost *et al.,* 1987; Gillen *et al.,* 1988; Stein *et al.,* 1992; Stein *et al.,* 1993) and that the severity of oesophageal injury is proportional to the degree of acid and pepsin reflux. In vitro studies have shown that acid can effect the proliferation and differentiation of the cells depending on the pattern of exposure. While prolonged exposure can lead to differentiation of BE cells, intermittent exposure can lead to proliferation of the cells (Fitzgerald *et al.,* 1996). The role of pepsin alone in the pathogenesis of BE has not been explored extensively in Barrett oesophagus. In fact it is difficult to separate the role of pepsin alone as activation of pepsin needs a pH of less than 3. Development of BE cannot be attributed to acid reflux alone. Studies have shown that the degree of oesophageal injury does not always correlate with the degree and frequency of acid reflux. BE is probably the consequence of a complex interaction between acid reflux, genetic susceptibility, environmental factors, and DGER. The fact that acid suppression does not prevent or reverse BE goes against the theory that acid could be the sole primary factor in the development of BE.

1.232 ROLE OF BILE ACIDS

There is increasing evidence that bile plays an important role in the development of BE (vide chapter 4 sec 4.15 for detailed discussion on bile acids). Studies have shown that patients with BE have increased bile reflux compared to patients with uncomplicated GERD. The fact that patients develop recurrent or progressive disease despite maximal acid suppressive therapy suggests that there must be other factors involved in the development of BE (Sontag 1992; Champion *et al.,* 1994; Jankowski *et al.,* 2000).

Complete regression or reversal of BE following acid suppression therapy has not been reported (Neumann *et al.,* 1995).

Oesophagitis has been seen in achlorhydric patients with pernicious anaemia (patients with lack of hydrochloric acid in gastric juice) (Palmer 1960; Orlando *et al.,* 1973). It has also been documented in patients with gastrectomy and oesophago-jejunostomy (anastomosis or joining of the oesophagus with the small bowel after removal of the stomach) (Nakayama 1956; Helsingen 1961; Meyer *et al.,* 1979) . Neoplastic progression of BE has been seen in patients who had bile reflux without any pathological acid reflux (Jankowski *et al.,* 2000). Bile acids are also involved in the promotion of goblet cell metaplasia in other gastrointestinal epithelium such as stomach, duodenum, intestine and bile ducts (Jankowski *et al.,* 2000). All these factors suggest that bile acids alone could be highly important in the pathogenesis of BE and its complications. The role of bile in the pathogenesis of BE is discussed in detail in chapter four which deals with evaluation of the cytotoxicity and genotoxicity of the bile acids.

1.24 MANAGEMENT OF BARRETT'S OESOPHAGUS

The management of BE is controversial. The goals of treatment in patients with Barrett's oesophagus are to eliminate symptoms of gastroesophageal reflux disease (GERD), heal erosive oesophagitis, reverse the metaplastic mucosa, prevent complications and detect dysplasia at an early stage. The final goal is to reduce the risk of progression to adenocarcinoma. These goals can be achieved by active management of gastro-oesophageal reflux and surveillance for malignancy. Patients with BE are known to have more reflux as compared to patients with uncomplicated GERD. Aggressive medical

management (i.e. pharmacological acid suppression) and possibly surgical fundoplication (surgical procedure which involves the wrapping of the upper part of the stomach around the lower end of the oesophagus to prevent reflux) are important components of the overall treatment of patients with Barrett's oesophagus.

1.241 MEDICAL MANAGEMENT

Medical treatment involves the use of histamine-2 receptor blockers and proton pump inhibitors (PPI). The pharmacological agents do not prevent reflux but suppress gastric acid production and thereby reduce the volume and frequency of reflux. The goal of the treatment is to raise the intra-gastric pH above 4 during the periods of the day when reflux occurs (Hunt 1999). H_2 blockers work by blocking the histamine stimulation of parietal cells, thereby reducing basal and, to a minor degree, postprandial acid production. The doses of H_2 blockers required to control GERD symptoms and heal oesophagitis are two to three times higher than those needed for the treatment of peptic ulcer disease. The H_2 blockers are found to be about 50% effective in healing and controlling the symptoms (Sabesin *et al.,* 1991; Sabesin SM 1991). Various PPIs like esomeprazole magnesium, lansoprazole, omeprazole, pantoprazole sodium, and rabeprazole sodium have been used in the management of GERD. The PPIs irreversibly bind and inhibit the H+, K+-Adenine Triphosphatase (ATPase) in the canalicular membrane of the parietal cells. The H+, K+ Adenine Triphosphatase (ATPase) proton pump is the final common pathway through which most stimuli act to induce the secretion of the gastric acid. At the recommended doses they are all found to have similar pharmacologic activity (Sachs 1997). Large controlled studies, have shown that one of the newer PPI, esomeprazole has significantly higher healing rates and symptom relief in

patients with erosive oesophagitis, compared with omeprazole, lansoprazole and pantoprazole (Labenz *et al.,* 1997). The PPIs are found to be more effective in treatment of oesophagitis with faster healing rates compared to the H2-blockers (11.5% per week versus 6.4% per week for H_2 blockers) (Klinkenberg-Knol *et al.,* 1994; Chiba 1997). Maintenance therapy with PPIs is often required at the same dosage as used for oesophageal healing in order to prevent recurrence (Vigneri *et al.,* 1995). Use of these pharmacological agents has been shown to be effective in relieving symptoms and healing oesophagitis (Hallerback *et al.,* 1994; Sampliner 1994). Studies conducted by Sampliner have shown that patients can still have abnormal 24hr pH in spite of treatment with high doses of PPI (Sampliner 1994). Studies have shown that long term treatment with PPIs can cause partial regression of BE (Peters *et al.,* 1999; Weston *et al.,* 2000). Complete regression of BE following long-term medical treatment is very rare and has not shown to alter the risk of progression to BE (Cooper 1982; Sharma *et al.,* 1997; Wilkinson *et al.,* 1999).

A number of prokinetic agents (bethanechol, metoclopramide, and cisapride) have also been used in the treatment of GERD. These agents act by either increasing lower oesophageal sphincter pressure, enhancing gastric emptying or by improving the peristalsis. One of these agents, cisapride, has been found to have similar efficacy to H_2 blockers in the treatment of mild oesophagitis when used as the primary agent and some facilitating effect when used in conjunction with these drugs (Galmiche *et al.,* 1988; Richter 1989; Hatlebakk *et al.,* 1997). More recently NSAIDS (COX-2 inhibitors) have been used in the treatment and chemoprevention of BE. The use of NSAIDS is based on the evidence that COX-2 is over-expressed in patients with BE and OADC. Studies have

demonstrated that use of a COX-2 inhibitor normalizes not only proliferation in vitro and in vivo, but COX-2 over expression is normalized as well (Buttar *et al.,* 2000; Kaur BS 2002). It has been shown that specific COX-2 inhibitors can decrease the incidence of cancer in animal models (Buttar *et al.,* 2002). Studies have shown that use of NSAID's can bring about 38%–90% reduction in the relative cancer risk (Thun *et al.,* 1993; Funkhouser *et al.,* 1995; Farrow *et al.,* 1998; Langman *et al.,* 2000). Better designed studies are required to prove the effectiveness of the COX-2 inhibitors.

1.242 SURGICAL MANAGEMENT

Unlike medical management which aims at reducing the gastric acid secretion surgical management aims at restoration of the lower oesophageal sphincter. Surgical management consists mainly of fundoplication and can be performed either laproscopically or as an open procedure. The surgical procedures aim at providing an appropriate length of intra-abdominal oesophagus, repairing the hiatal defect, and creating a loose, "floppy" fundoplication. Current experience with anti-reflux surgery suggests that there is no one best operation for all patients. Factors such as the degree of oesophageal shortening, disturbances of oesophageal motility, prior operations, and surgeon's skill decide the type of operation. Studies have shown that surgical procedures tend to offer a superior control of reflux compared to medical management (DeMeester *et al.,* 1986; Spechler 1992; Peracchia *et al.,* 1995). Correctly performed anti-reflux surgery early during the course of GERD prevents the development of BE (DeMeester 2002). Although there are considerably more reports of regression following anti-reflux surgery, regression is rarely complete and occurs in only 10–44% of patients (Caygill *et al.,* 2004).

Although surgical therapy has been found to have slightly better results than medical therapy, neither of them has shown to have a major impact on the progression of BE to adenocarcinoma. While some people ((Low *et al.,* 1999; Hofstetter *et al.,* 2001; Oberg *et al.,* 2001) believe that fundoplication causes regression of Barrett's there are others who believe that fundoplication has no effect on the reversal of Barrett's (Cheu *et al.,* 1992; McDonald *et al.,* 1996; Csendes *et al.,* 1998; Gutschow *et al.,* 2002). Some people believe that the refractory nature of the disease could be due to ongoing reflux, especially bile reflux, which may contribute to metaplastic changes (DeMeester 2002). There are reports of high grade dysplasia and adenocarcinoma after surgery and evidence that there is an increased rate of failure of anti-reflux surgery in patients with BE compared to patients with uncomplicated GERD (Williamson *et al.,* 1990; Little 1994; Csendes *et al.,* 1998). This could probably be due to the contribution of other agents such as bile acids in the pathogenesis of BE. Owing to this Csendes et al have tried more aggressive antireflux procedures such as duodenal switch operation (Csendes *et al.,* 1998). More recently Csendes *et al.,*(Csendes *et al.,* 2002) found that surgery of acid suppression and bile diversion (i.e. truncal vagotomy with anterectomy and Roux-en-Y gastrojejunostomy with fundoplication) had the best results in patients with recurrent reflux after surgery.

1.243 NEWER TECHNIQUES FOR ERADICATION OF BARRETT'S

A number of new endoscopic reversal techniques have been used for the reversal and eradication of BE. These methods involve selective and complete ablation of BE leaving the underlying layers intact whilst trying to keep the pH above 4 by the simultaneous use of PPI's or by anti-reflux surgery (Barbezat 2000; Sharma 2001). Various ablation

techniques include thermal coagulation (multipolar electrocoagulation, bipolar electrocoagulation, heater probe, argon beam plasma coagulation) lasers (Nd:YAG and KTP), photodynamic therapy and endoscopic resection (Lim *et al.,* 1999). Regression of BE has been shown with ablation techniques with squamous re-growth. However evidence of metaplastic islands buried underneath the squamous undergrowth was found by some of the investigators (Berenson *et al.,* 1993; Barham *et al.,* 1997). The endoscopic techniques include photodynamic therapy (PDT), laser photocoagulation, argon plasma coagulation electrocoagulation and cryotherapy.

Photodynamic therapy is a non-thermal chemical ablation method which is based on the action of visible light on a previously administered photosensitizer which has been taken up by the tissue. The photo sensitizers used in Barrett's include hematoporphyrin derivative (HpD) or Photofrin (porfimer sodium) and 5-amonolevulinic acid (ALA). ALA has been found to be ideal in Barrett's metaplasia because its intermediate compound protoporphyrin IX (PpIX) which is a photoactive compound is selectively produced by oesophageal mucosa (Gossner *et al.,* 1998). ALA can be given orally and produces superficial damage with minimal scarring and subsequent stricture formation. Lasers are the most commonly used light source in the PDT. This is normally delivered by fiber placed endoscopically in the lumen of the oesophagus. Varying degrees of eradication of BE with re-epithelialisation has been reported with PDT. Studies have shown islands of buried columnar glands in patients with re-epithelialisation (Kelty *et al.,* 2002). Owing to the short follow-up periods these results have to be viewed with caution.

Endoscopic mucosal resection has been used in the staging and treatment of patients with Barrett's oesophagus (Soehendra *et al.,* 1997; Ell 2000). This has been used in

management of early localised cancers. Further studies and long-term follow up are required before any conclusive remarks can be made about this technique.

Most of the mucosal ablative methods are in experimental stage and none of them have been shown to reduce or eliminate the cancer risk.

1.244 ENDOSCOPIC SURVEILLANCE OF BARRETT'S

Endoscopic surveillance forms one of the main components of management of patients with Barrett's metaplasia. It is based on the assumption that BE adversely affects survival and that surveillance can detect early and curable cancers. These assumptions are controversial with a lack of strong evidence to support them. Though surveillance can detect early cancers its reliability and efficacy in improving survival from oesophageal cancer remains unclear (Spechler 2000). Sampling error is one of the factors which adversely affect endoscopic surveillance. The sensitivity of endoscopy could be increased by using the protocol of taking four quadrant biopsies at 2 cm interval with a jumbo biopsy forceps (Cameron *et al.,* 1997). A number of newer techniques such as chromoendoscopy, endosonography and fluorescent techniques have been used to detect BE in-spite of which sampling error still remains a problem. Another factor which adversely affects surveillance is the fact that the prevalence of symptomatic GERD is high in the general population while the prevalence of BE is very low in patients with symptomatic GERD. Hence only a very small percentage of patients (5%) with BE in the general population are detected by endoscopy whilst vast majority of patients with BE (95%) remain undetected due to asymptomatic GERD (Sontag 2001). Surveillance aims at reducing morbidity and mortality by treatment and early detection of BE and treatment of high grade dysplasia and oesophageal adenocarcinoma. . The current BSG guideline

recommends that when surveillance of nondysplastic CLO (columnar line epithelium) is considered appropriate, it should be performed every 2 years (BSG Guidelines in Gastroenterology, Aug 2005). Patients with LGD are managed by extensive acid suppression for 8-12 weeks followed by extensive re-biopsy. If the changes persists surveillance should be carried six monthly. Persistent HGD may be associated with invasive adenocarcinoma in 30-40% of the patients. If the diagnosis is confirmed by two pathologists and the patient is fit oesophagectomy is recommended. In those unfit for surgery endoscopic ablation or mucosal resection should be considered (BSG Guidelines in Gastroenterology, Aug 2005).

1.3 GENETIC CHANGES IN BARRETT'S

Below is a brief overview of the cell cycle followed by summary of the main candidate genes implicated in cancer development in patients with BE.

1.31 CELL CYCLE AN OVERVIEW

Knowledge of the cell cycle and its control is central to the understanding of most carcinogenic processes. A brief overview of the cell cycle and its control and the various stages affected in Barrett's oesophagus is given below. The cell cycle is the process through which the cells grow and duplicate themselves. This involves the accurate duplication of DNA and their faithful distribution to the two daughter cells. The Mammalian Cell Cycle consists of four discrete phases namely G1, S, G2 and M phase (see figure 1.3).

G1 Phase (Gap-1):

G1 phase is characterised by cell growth and protein synthesis. This is the only part of the cell cycle regulated by external stimuli (growth factors and mitogens).

S Phase (synthesis phase):

This is the phase of DNA replication. After completion of DNA replication the cell enters the G2 phase.

G2 phase:

During this phase the cell undergoes growth and protein synthesis.

M Phase:

Mitosis is characterised by the following stages (see figure 1.4): -

Prophase:

During prophase the chromosomes condense and become visibly distinct. Prophase ends when the nuclear envelope breaks down, abolishing the distinction between nucleus and cytoplasm. By late prophase the two chromatids move towards the poles and form a mitotic spindle.

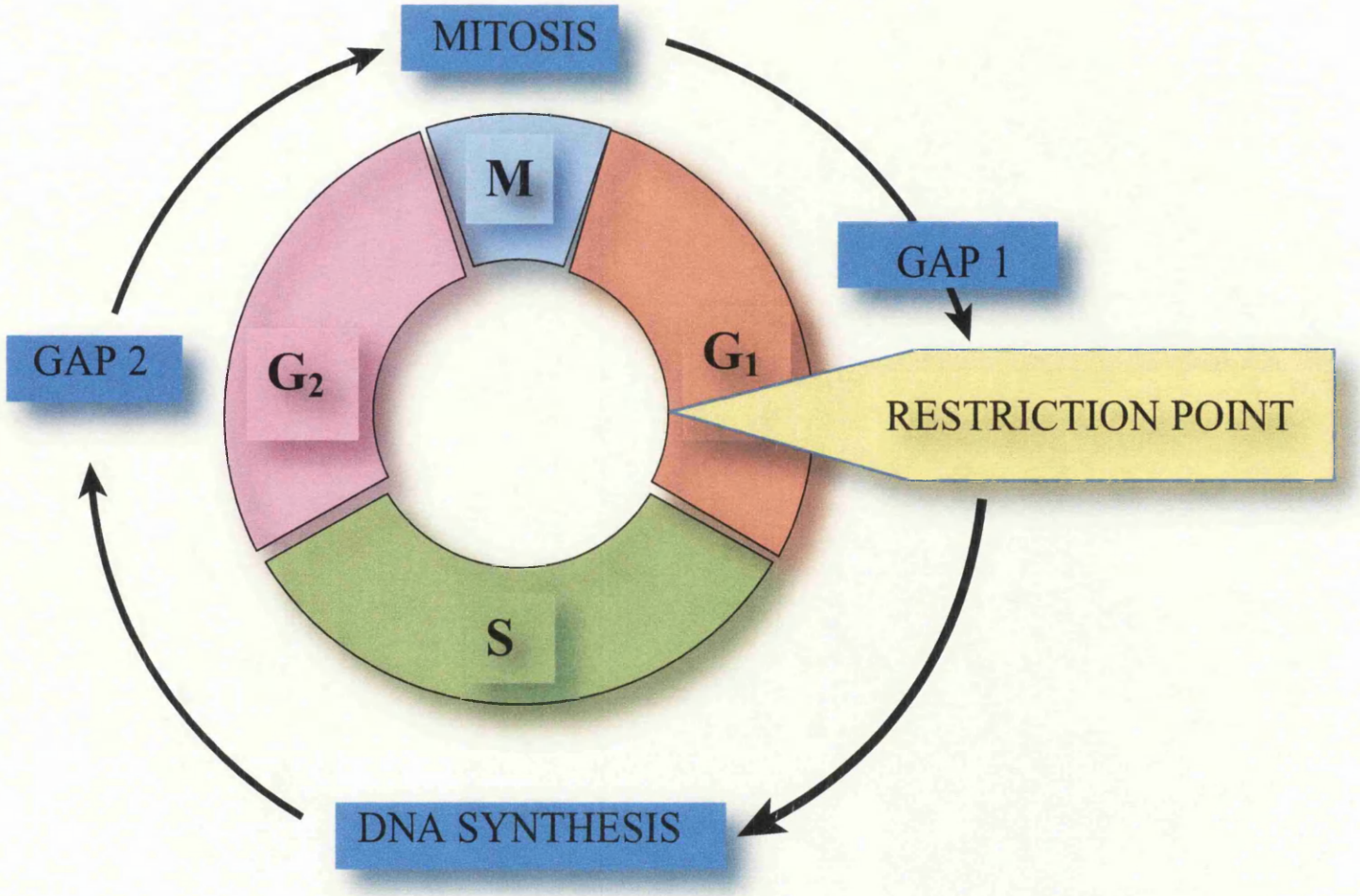
Metaphase:

The discrete chromatid pair takes places in the centre of the mitotic spindle. The chromatids are tightly coiled and discrete.

Anaphase:

During this phase the sister chromatids separate and move to the opposite poles.

FIGURE 1.3 SHOWING THE PHASES OF THE CELL CYCLE



The cell cycle is grouped into four periods: S (synthesis), G2 (second gap), M(mitosis) and G1 (first gap). Interphase refers to the three phases S, G1 and G2. Modified from Alberts et al., 2002

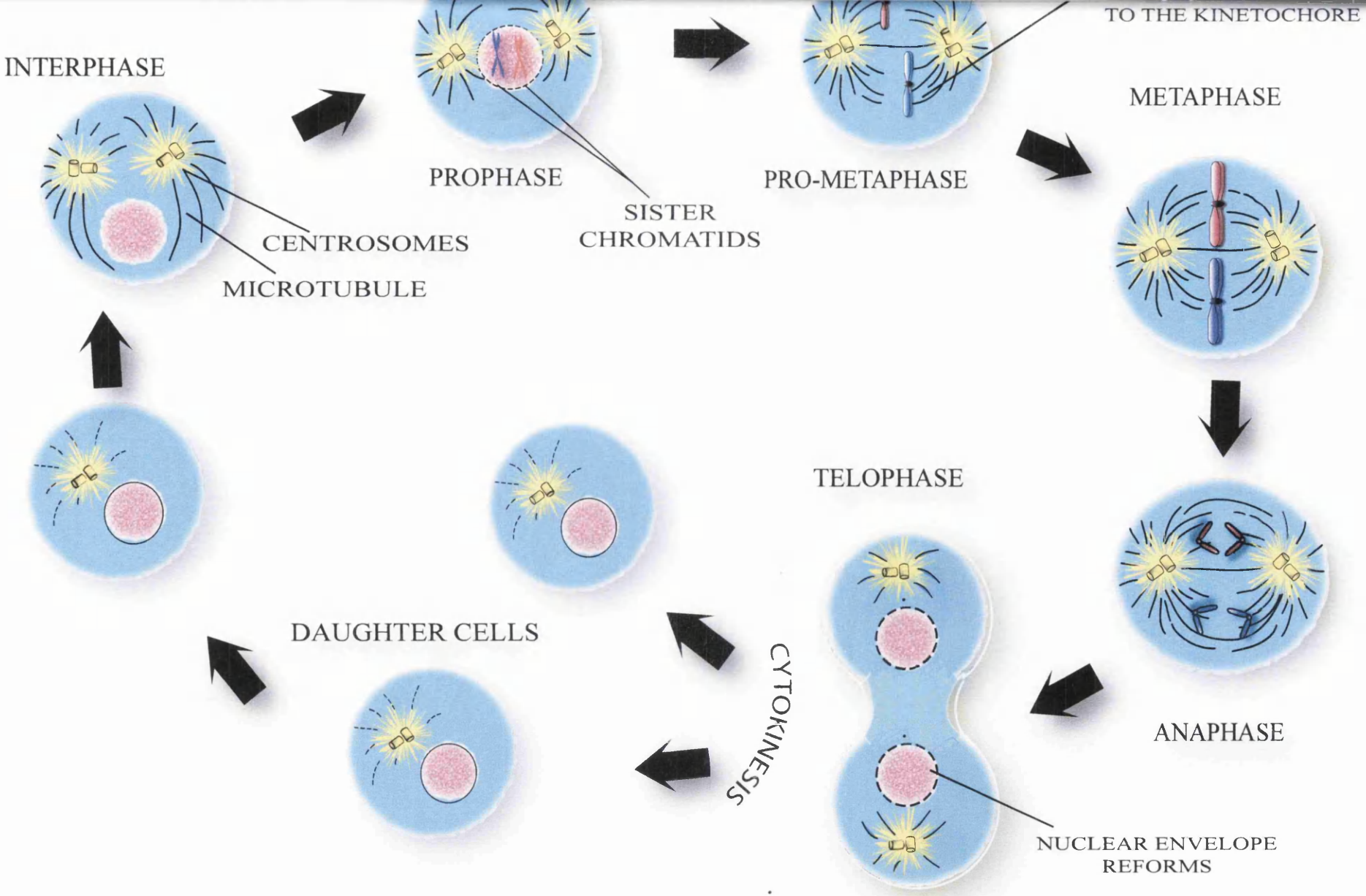


FIGURE 1.4 SHOWING THE STAGES OF MITOSIS
(Adapted from Alberts et al., 2002)

Telophase:

Telophase is characterized by reconstruction of the nuclear membrane around each daughter nucleus. This is followed by cytokinesis which is the division of the cytoplasmic part of the cell. In rapidly replicating human cells the full cycle lasts about 24 hours, with G1 9 hours; the S phase 10 hours; G2 phase 4.5 hours and mitosis about 30 minutes (Lodish *et al.,* 2000).

1.32 CANCER AND CONTROL OF CELL CYCLE

Cancer cells arise from normal cells by progressive acquisition of mutations over a period of time. These multiple genetic alterations can involve genes that control various aspects of cell proliferation, differentiation and apoptosis [reviewed by (Bertram 2000; Bell *et al.,* 2005). Mutations can lead to activation of genes which stimulate cell proliferation or inhibit apoptosis (Oncogenes) or inactivation of genes which normally inhibit cell proliferation (tumor suppressor genes). Uncontrolled cell proliferation is the hallmark of cancer. Loss of cell cycle control is the major defect encountered in most cancers. Many of the genes mutated in human cancers are directly involved in the regulation of cell cycle [reviewed by (Bell *et al.,* 2005)]. The point between the early G1 and late G1 phase that represents an irreversible commitment to undergo one cell division is termed the **restriction point**. The restriction point divides the cell cycle into a mitogen dependant (growth factor), early G1 phase and a growth factor independent phase (late G1 through mitosis). Oncogenic processes exert their greatest effect by targeting particular regulators of G1 phase progression. G1 progression depends on stimulation by mitogens. Passage

through the restriction point and entry into S phase is controlled by cyclins, cyclin dependant kinases (Cdks) and the Rb protein.

1.321 THE RETINOBLASTOMA GENE

The key player in G1 phase cell cycle control is the Rb protein, which is the product of retinoblastoma the gene. **Rb gene** was the first tumour suppressor gene to be identified well over a decade ago (Friend *et al.,* 1986). The Rb protein is a 928 amino acid phospho-protein, which arrests the cells in the G1 phase (Weinberg 1995). In cycling cells the retinoblastoma protein (pRb) is un– and/or hypo–phosphorylated in early G1 and becomes hyper–phosphorylated (inactive form) on late G1. The hypo-phosphorylated Rb is the active form, which suppress cell proliferation in G1 phase. Phosphorylation of Rb inactivates the growth suppressive function of Rb allowing the cells to progress through the restriction point [reviewed by (Harbour *et al.,* 2000; Mittnacht 2005)]. The Rb protein forms complexes with the E2F family of transcription factors and inhibits the transcription of E2F regulated genes (see figure 1.5). E2F consists of a heterodimeric complex of an E2F polypeptide and the DP protein. The phosphorylation of Rb results in its dissociation from E2F leading to activation of E2F target genes. E2F is a potent stimulator of cell cycle entry. E2F target genes include the ones required for G1 cycle progression and DNA synthesis such as:

- C-myc, B-myb, Cyclin E, Cdc2 and those encoding thymidine kinase, dihydrofolate reductases, DNA polymerase.
- P19ARF (involved in apoptosis)

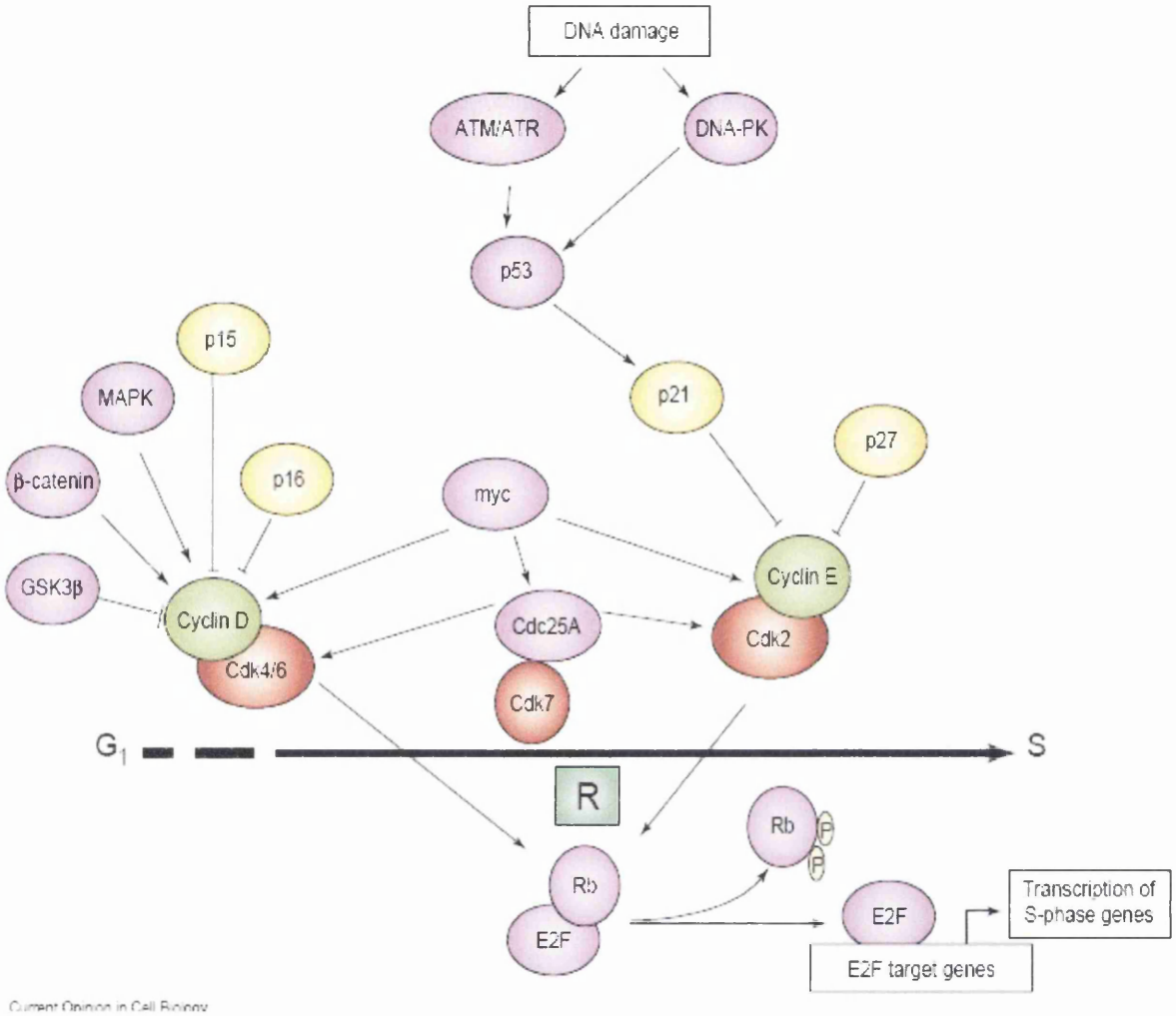


Figure 1.5 Mechanisms controlling S-phase initiation in animal cells. The cyclin/cdk complex becomes activated by phosphorylation in specific residues of the catalytic subunit of the cdk by another cdk, cdk7/cyclinH, also known as'cdk activating kinase' (CAK) (red).These complexes are in turn regulated by a stoichiometric combination with small proteins. These proteins, called cyclin-dependent kinase inhibitors(CKIs), include p16ink4a, p15ink4b, p18ink4c, p19ink4d, p21waf1, p27Kip1 and p57kip2(yellow). DNA synthesis (Sphase) occurs when Rb is phosphorylated by active cdk4 and/or cdk6 releasing the transcriptional factor E2F from Rb allowing cells to progress to Sphase. Arrest of cells at G1 following DNA damaging agents are due to the transcriptional activation of the tumor-suppressor gene p53, leading to the up regulation of p21, there by inhibiting cdks. **R,** Restrictionpoint. Adapted from Senderowicz *et al.*, 2004.

Thus activated E2F promotes G1-S transition and DNA synthesis through transcription factors. The phosphorylation of Rb is brought about by Cdk/cyclin D complexes (blocked by p16 CDK inhibitor). The E2F's are regulated by two pRb family members – p130 and p107 during G0 and S phase respectively.

Allelic loss of the Rb gene occurs late in the metaplasia-dysplasia-carcinoma sequence in BE (Sarbia M 2001). About 50% of Barrett's adenocarcinoma's show loss of the 13q allele (Boynton *et al.,* 1991). Decrease in the size of Rb m-RNA transcript and quantity have also been seen in patients with Barrett's dysplasia and cancer (Huang *et al.,* 1993). There are no studies which have demonstrated mutation of Rb gene either in Barrett's metaplasia or Barrett's adenocarcinoma.

1.322 CYCLIN-CDK COMPLEXES

Entry into the S phase of cell cycle depends on the activation of cyclin dependent kinases(CDKs).**Cyclins** are a diverse family of proteins which bind and different members of the CDK family [reviewed by (Evan *et al.,* 2001; Sanchez *et al.,* 2005)]. The concentration of most cyclins changes during various stages of the cell cycle, resulting in oscillations of CDK activity that forms the foundation of the cell-cycle control system. The regulation of cyclin concentration is primarily by changes in cyclin gene expression and cyclin proteolysis, and hence is of fundamental importance in cell-cycle control.

Cyclins D1 and E are proto-oncogenes. Over expression of these cyclins leads to phosphorylation and inactivation of p105-Rb.Amplification of cyclin D1 gene (located in **11q13)**. Arber *et al* showed that there was an increased nuclear expression of cyclin D1 in patients with Barrett's oesophagus (34%) compared to normal squamous controls (Arber *et al.,* 1996). Recent studies have shown that amplification cylcin D1 occurs in early stages of barrett's and is found to correlate negatively with survival (Miller *et al.,* 2003; Trudgill *et al.,* 2003). Overexpression of cyclin E has not been found in early stages of BE and low grade dysplasias. However, Sarbia *et al* showed cyclin E was over-expressed in 10% of cases with low grade dysplasia, 18% of those with high-grade dysplasia, and in 14% of adenocarcinomas in Barrett's oesophagus (Sarbia *et al.,* 1999).

1.323 CDK REGULATION BY CDK INHIBITORS (CKIs)

The activity of CDK is extensively regulated by Cdk inhibitor proteins (CKIs) that bind and in-activate various Cdks and cyclin-Cdk complexes.Based on their structures and affinities two families of CKIs have been identified. Members of the Cip/Kip family(p21, p27, and p57) inhibit CDK2 while members of the INK family(p15, p16, p18) inhibit CDK4 and CDK6 (Sherr *et al.,* 1999; Vidal *et al.,* 2000).

1.324 THE p16 GENE (*INK4a/ARF*)

The p16 gene is located on chromosome 9p21 and belongs to the INK4 family of CKIs. The p16 gene encodes the CDKI, which regulates progression through G1/S of the cell-

cycle by binding CDK 4/6, and so limits CDK 4/6–cyclin D complex formation and phosphorylation of Rb. This locus also encodes p14ARF, which functions to sequester MDM2 and inhibit p53 degradation (Sharpless 2005). p16 and p14ARF have unique promoters and first exons but share common second and third exons. Alteration in the shared exon region can affect the function of both genes simultaneously. Alterations of p16 are reported in various human malignancies, exceeded in frequency only by the p53 tumor suppressor gene. Loss of p16 basically mimics cyclin D1 over-expression. Homozygous deletion, point mutation, and hypermethylation of the promoter region of p16 have been described (Barrett *et al.,* 1996; Wong *et al.,* 2001).

1.33 INVOLVEMENT OF MITOGENIC SIGNALLING PATHWAYS

1.331 C-erbB2 (HER-2/neu) and EGFR

The **C-erbB2** (also called HER-2/neu) is located on the chromosome **17q21** and encodes for the epidermal growth factor receptor (EGFR) which belong to subclass 1 of the receptor tyrosine kinase superfamily (Normanno *et al.,* 2006). The ErbB receptors and their ligands play a fundamental role in development, proliferation, cell differentiation and carcinogenesis. CerbB2 and its ligands have been implicated in the development of OADC and BE (Jankowski *et al.,* 1992). The amplification of this gene occurs at a late stage (Nakamura *et al.,* 1994). In one study, 10% of adenocarcinomas and 31% of high grade dysplasias in Barrett's oesophagus exhibited erbB-2 overexpression (Kim *et al.,* 1997). Overexpression of erbB-2 in oesophageal adenocarcinomas has been associated with a poor outcome and a poor overall survival rate (Brien *et al.,* 2000).

1.332 TRANSFORMING GROWTH FACTOR β SIGNALLING

Transforming growth factors are a large family of highly conserved growth regulatory polypeptides which govern a number of biological activities including cellular growth, differentiation and adhesion (de Caestecker 2004). Its effects on cell growth vary depending on the cell type and growth conditions and could be either stimulatory or inhibitory [Reviewed by (Massague *et al.,* 2000; de Caestecker 2004)]. TGF-β plays an essential role in a wide array of cellular processes. Transforming growth factor beta (TGFβ) is a potent anti-proliferative agent acting through two receptors (TGFβRI and TGFβRII) which transduce downstream signals via cytoplasmic transcription factors called Smad proteins (derived from Sma- and Mad- related protein). The loss of function of TGFβ signalling in Barrett's associated adenocarcinoma could be related to Smad 4 mutations (Lebman *et al.,* 2002; Onwuegbusi *et al.,* 2005) or to the loss of mRNA expression of TGFβRII (Garrigue-Antar *et al.,* 1996).

1.34 TUMOR SUPPRESSOR GENES

1.341 THE ROLE OFTHE p53 TUMOUR SUPPRESSOR GENE

p53 was the second tumour suppressor gene to be characterised after Rb and is located on the short arm of chromosome 17 (17p13). It is the most commonly mutated gene in most human cancers (Sherr *et al.,* 2000; Yee *et al.,* 2005). Activation of p53 results in increased transcription of genes involved in DNA repair, G1 arrest and apoptosis. The p53 protein is a transcription factor that enhances the rate of transcription of six or seven genes. The human p53 protein contains 393 amino acids. The activation of p53 is negatively regulated by the MDM2 protein (see figure 1.6).

PRODUCTS OF GENES TRANSCRIPTIONALLY ACTIVATED BY P53

P21,WAF1, Cip1	Inhibits several cyclin-cyclin dependent kinases.
MDM2	product of an oncogene; inactivates p53-mediated transcription
GADD45	Induced upon DNA damage, binds to PCNA and can arrest the cell cycle. It is is involved in the DNA nucleotide excision repair
CYCLIN-G	Novel cyclin of unknown function and no known cyclin dependent kinase.
BAX	A member of the BCL2 family that promotes apoptosis.
IGF-BP3	The insulin like growth factor binding protein 3; blocks signalling of a mitogenic growth factor.

The p53 protein is normally kept at a low concentration by its short half life (about 20 minutes). p53 is stimulated by several types of DNA damage and hypoxia. p53 is also activated when the ribonucleoside triphosphate pool falls below a critical threshold. DNA damage activates the ATM protein (mutated in ataxia telangiectasia) which in turn can activate p53 either by direct (phosphorylation) or indirect means (initiates phosphorylation cascade). p21, which is one of the downstream transcription products binds to a number of cyclin and cdk complelxes and inhibits phosphorylation. It also binds to PCNA (Proliferating Cell Nuclear Antigen). p21 by its interaction with cyclins and PCNA stops DNA replication (Meek 2004).

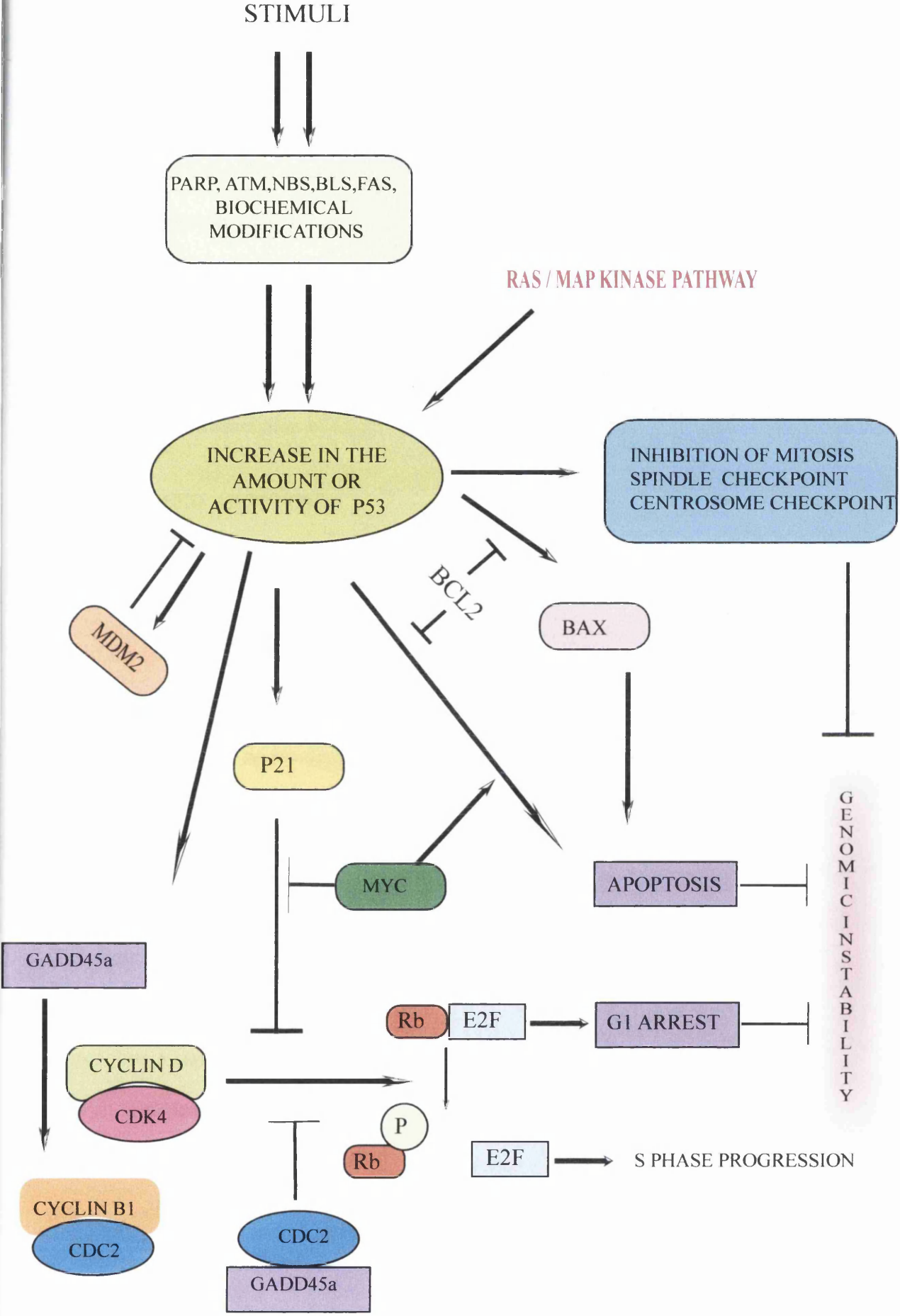


FIGURE 1.6 SHOWING THE COMPONENTS OF P53 SIGNALLING PATHWAY
(Adapted from Agarwal et al., 1998)

Transcription of Gadd45 (growth arrest and DNA damage–inducible) by activated p53 produces a protein which can recognize an altered chromatin state and cause de-stabilisation of histone-DNA interactions by interacting directly with the four core histones. This enables increased acessability of damaged DNA to proteins involved in repair processes. Gadd45 also causes G2 arrest by direct interaction with Cdc2 proteins which results in dissociation of Cdc2-cyclinB1 complex.

1.3411 P53 AND APOPTOSIS

A number of factors affect the decision of the cell to enter the p53 mediated cell cycle arrest or apoptotic pathway [reviewed by (Hengartner 2000; Hickman *et al.,* 2002)]. When the genomic damage is significant and non-repairable, p53 levels continue to rise til caspases cascade leading to apoptosis.Recent studies have also shown that p53 can induce apoptosis by translocation into the mitochondria (Murphy *et al.,* 2004).

p53 Alterations (mutation, LOH) occurs later in the disease progression and is associated with increased risk to progression to OADC (Paulson *et al.,* 2004; Novotna *et al.,* 2006).

P53 mutations frequently occur in BE [(with mild or no dysplasia) 30-66%] and OADC (40-88%) (Doak *et al.,* 2003). Mutated p53 molecules have an altered conformation and do not form a complex with MDM2 and are therefore not degraded and accumulate inside the cell. This is detected by immunohistochemistry and is seen in LGD (7%), HGD (30-60%) and OADC (45-85%) but not in patients with BE (Doak *et al.,* 2003). LOH of 17p (the p53 locus) increased from 6% in patients without dysplasia to 57% in HGD (Reid *et al.,* 2001). Accumulation of p53 protein was found to be common in patients with BE (Segal *et al.,* 2004). However studies have shown that p53 accumulation seen detected

by immunohistochemistry does not correlate with presence of p53 mutation and the exact mechanism for accumulation of p53 protien is yet to be determined (Doak *et al.,* 2003).

1.3412 MDM2 (MURINE DOUBLE MINUTE-2 GENE)

MDM2 negatively regulates p53 in several ways (Levine 1997; Murphy *et al.,* 2004). Firstly its binding with P53 interferes with p53's ability to transactivate target genes. Secondly MDM2 has an intrinsic ubiquitin ligase activity which contributes to p53 degradation. Thirdly it relocalizes p53 from the nucleus to the cytoplasm where it undergoes proteosomal degradation. MDM2 is inhibited by ARF (p19 ARF). ARF can antagonize any or all of the adfore mentioned functions of MDM2. Therefore, activated ARF keeps p53 induced, active, mostly confined to the nucleoplasm and hence stable. MDM2 amplification has been seen in patients with oesophageal adenocarcinoma (Morgan *et al.,* 1999). Soslow *et al* (Soslow *et al.,* 1997) found that significant expression of MDM2 occurred only in cases with wild-type p53, whereas the cases with mutated p53 showed little if any expression of MDM2. Studies have shown increased expression of MDM2 in areas specialised intestinal metaplasia compared to normal squamous mucosa (Fujii *et al.,* 2003).

1.342 RAS AND CELL CYCLE REGULATION

Ras genes are among the most frequently activated oncogenes in cancer. They were the first oncogenes to be implicated in human cancer. Mammalian cells encode three functional ras genes: H-ras, K-ras and N-ras all of which show a similar structure and function [Reviewed by (Takuwa *et al.,* 2001; Cox *et al.,* 2002)]. The Ras proteins are proto-oncogene products that are critical components of signalling pathways leading from cell surface receptors to the control of cellular proliferation, differentiation or cell death. K-ras mutation is common in several human cancers. K-ras codon 12 mutation is found in about 70% of pancreatic cancers and 50% of colorectal cancers. Role of ras in progression of BE is uncertain. Mutation of codon 12 of kras was seen in BE (0.4%), dysplasia (4%) and oesophageal cancer (36%) (Trautmann *et al.,* 1996). K-ras mutation appears to be a late event in the progression of BE to OADC although no correlation has been established with prognosis or evolution of the disease (Kyrgidis *et al.,* 2005).

1.343 THE APC GENE

The APC gene (Adenomatous polyposis coli gene) is located on chromosome 5q21. Germ line mutation in the APC gene results in familial adenomatous polyposis, which is a dominantly inherited disorder that predisposes patients to colorectal cancer. APC mutation is also seen in about 80% of sporadic colorectal cancers (Bienz *et al.,* 2004). The major function of APC is the negative regulation of β-catenin levels. β-catenin is involved in both the E-Cadherin cell adhesion system and the Wnt-1 signalling pathway. Studies have shown that APC gene mutation can occur in patients with oesophageal adenocarcinoma though it is not as common as in colon cancer (Kyrgidis *et al.,* 2005).

LOH of APC gene locus and hyper-methylation of APC gene has been reported in patients with BE with dysplasia (Bektas *et al.*, 2000; Kawakami *et al.*, 2000).

1.35 CHROMOSOMAL STRUCTURE

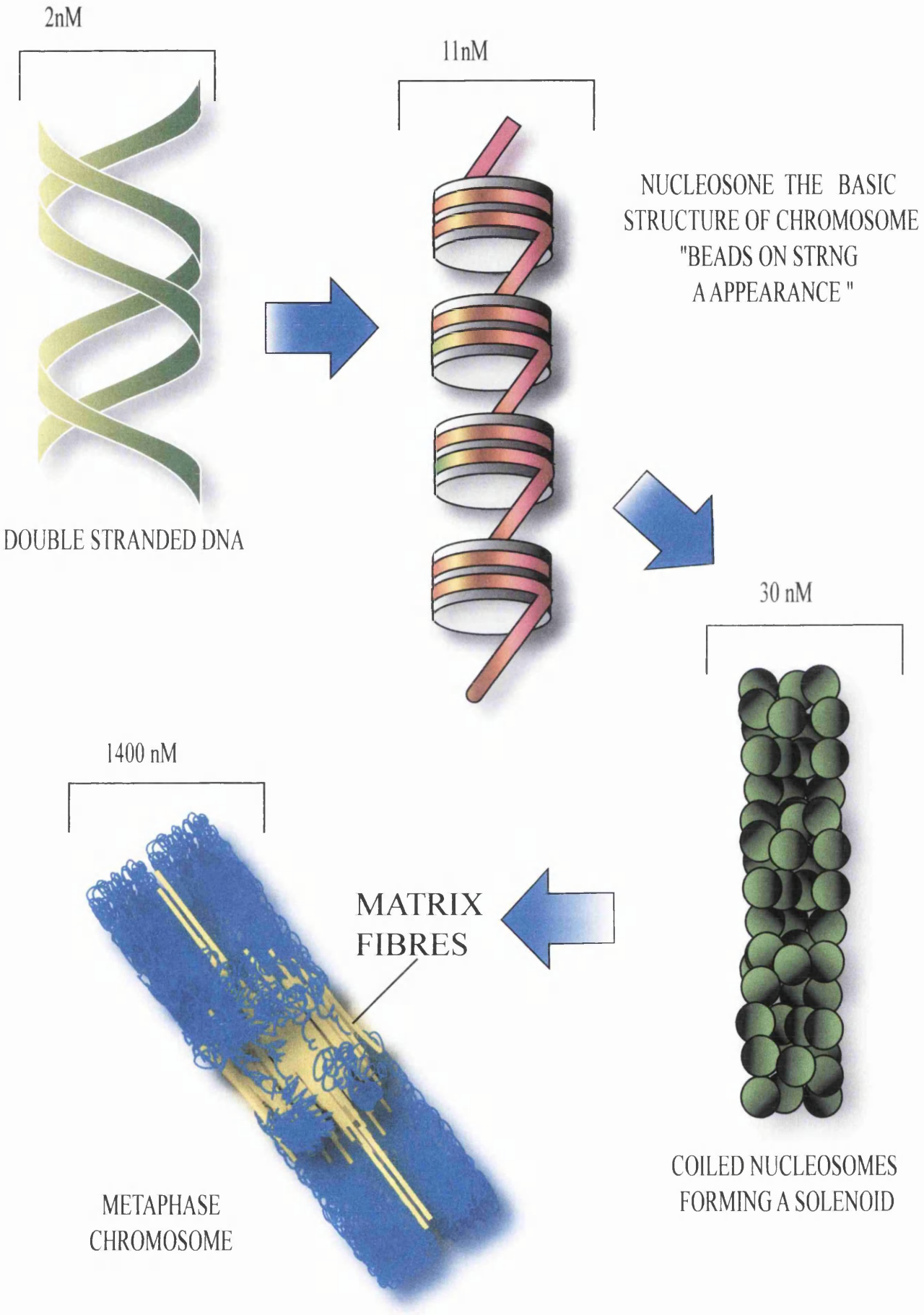
Chromosomes are distinct dense bodies found in the nucleus which are composed of protein and DNA. The DNA carries the genetic information of the cell. Each chromosome consists of a single, continuous linear double-stranded DNA molecule. The DNA molecule of the chromosome is found as a complex with a family of basic chromosomal proteins called histones and with a heterogeneous group of acidic, non-histone proteins. The complex of DNA and protein together are called chromatin. During most of the cell cycle (i.e. interphase) the chromosomes are less condensed and are not visible as individual objects under the light microscope. The chromosomes are visible during the metaphase of cell division as dark dense bodies as they become highly condensed. Five major histones are involved in the packaging of the chromatin (H1, H2A, H2B, H3 and H4). The basic unit of chromatin structure is called nucleosome. Each nucleosome consists of an octamere made up of two copies each of the four core histones namely H2A, H2B, H3, and H4 (see figure 1.7). The DNA double helix winds round the histone core making almost two turns. Approximately 140 base pairs of DNA are associated with each histone core and they are called the core DNA. The linker or the spacer DNA (20 to 60 base-pair) which is associated with H1 histone connects one nucleosome to the other. This gives chromatin the beads on a string appearance. The long strings of nucleosomes are further condensed into 30-nm-diameter cylindrical fibers called "solenoid" fibers". The solenoids are further packed into **loops** or domains attached at intervals to a nonhistone protein **scaffold** or matrix. During metaphase the

condensation starts at the centromere, which is composed mainly of matrix fibers, and ends at the telomeres.

1.35 CANCER AND CHROMOSOMAL MUTATION

Cancer is a genetic disease of the somatic cells and is characterised by deviations of the normal genetic mechanism which regulate the cell growth. Uncontrolled cellular proliferation is the hallmark of cancer cells. Carcinogenesis is a complex multi-step process in which a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells (Foulds 1954; Nowell 1976). It is well accepted that the fundamental features of cancer is tumour clonality, which involves the development of tumours from single cells that begin to proliferate abnormally. The initial step in carcinogenesis is called INITIATION during which a normal cell undergoes an irreversible genetic change which confers on it the capacity for autonomous growth. Initiation can occur following a single exposure to a carcinogen, and the changes, though irreversible, can remain latent for a long period of time (weeks to months or longer if promotion does not occur). The next step involves promotion in which the initiated cells, upon repeated exposure to specific agents (promoters), become expanded to a visible population (Cooper *et al.,* 1995). One or more of the clonally expanded initiated cells, as a result of additional mutations, evolve into a cancer cell. This cell again undergoes clonal expansion, and through further changes becomes progressively malignant. This process of development of an initiated cell into biologically malignant cell population is called tumour progression.

FIGURE 1.7 SHOWING THE STRUCTURE OF CHROMOSOME
Modified from Alberts et al., 2002



1.36 TYPES OF CHROMOSOMAL MUTATION

Chromosomal aberrations are common in cancer. David von Hansemann in 1890 was the first to describe the occurrence of aberrant mitoses in cancer cells. A few decades later Theodor Boveri in his *Zur Frage der Entstehung Maligner Tumoren* ("The Origin of Malignant Tumours" 1914) postulated that cancer originates as a consequence of disturbance in chromosomal balance. Chromosome aberrations could be either in the form of variation in chromosome number or variation in chromosome structure. Aberrations in chromosome structure occur following breakage of the chromosome. Chromosome breakage could be either spontaneous or induced by mutagenic agents such as ionizing radiation, ultraviolet light, viruses and chemical agents. The broken ends of a chromosome are sticky and tend to rejoin. Change in the chromosome structure can be in the form of deletions, duplication, translocation and inversion.

1.361 STRUCTURAL CHROMOSOMAL ABERRATIONS

1.3611 DELETIONS

Deletions can arise from either a single or double break in the chromosome. Double breaks can produce **interstitial deletion** while a single break can produce a **terminal deletion.** If the double break occurs in the different arm of the chromosome it can lead to the formation of a ring chromosome. Chromosome deletions can vary in size from few base pairs to many mega base pairs. The effect of deletion depends upon the size of the deletion. A small deletion within a gene, called an **intragenic deletion,** inactivates the gene. Larger deletions (**multigenic**) involving two to several genes can be lethal or have

very severe consequences. Homozygous deletions of essential segments can be lethal. Heterozygous deletions could be lethal or can uncover recessive alleles, causing them to be expressed phenotypically. An example is *cri du chat* syndrome caused by heterozygous deletion of the short arm of of chromosome 5 which is characterised by physical and mental retardation and striking cry that resembles that of a cat (Griffiths *et al.,* 2002). Deletion of tumour suppresor gene can lead to cancer. Deletion of 13q14 can lead to Retinoblastoma (Heim S 1995). Deletions are common in epithelial adenocarcinomas and are usually multiple in numbers.

1.3612 DUPLICATION

Duplication is an aberration in which a segment of a chromosome is repeated and thus is present in more than one copy within the chromosome. The chromosomal segment may be repeated side by side in an original order (tandem duplication) or in a reverse order (reverse tandem). The duplicated segments can be in a novel location on the same chromosome or on another chromosome. Duplications are generally rare and hard to detect. Most duplication consists of an extra chromosomal arm or part of an arm, generally attached to a non-homologous chromosome (Griffiths *et al.,* 2002). Duplications may or may not be lethal. Pallister Killian syndrome characterised by mental and physical retardation is associated with duplication of short arm of chromosome 12 (Adachi *et al.,* 2003). In fact duplications can provide additional genetic material which can carry out new functions (evolutionary significance).

1.3613 INVERSION

Inversion is a chromosomal mutation in which the chromosomal segment is turned around by 180°. Inversions can either be pericentric (when the inverted segment involves the centromere) or paracentric when the inverted segment involves the chromosome arm without including the centromere. Genetic material is not lost following inversion. If the break points occur within a gene or a region which controls gene expression then inversion can lead to phenotypic consequences. Though duplication and inversion are both rare, like translocation, it can cause abnormal juxtaposition of genetic materials resulting in the formation of chimeric genes producing abnormal proteins that can initiate neoplasia. Inversion of RET (rearranged during transfection) proto-oncogene on chromosome 10 leads to the formation of oncogene RET/*ptc* which is found frequently in the papillary carcinoma of the thyroid (Kufe, *et al.*, 2003).

1.3614 TRANSLOCATION

Translocation involves change in the position of the segment of chromosome and the gene sequence contained in it. Translocation does not involve loss or gain of genetic material. Translocation can occur within the same chromosome (intra-chromosomal translocation) or between two non-homologus chromosomes (inter–chromosomal translocation). If the transfer occurs in one direction it is called non-reciprocal translocation and reciprocal translocation when it involves exchange of segments between the two chromosomes. Reciprocal translocations are the commonest type of translocation. Translocations are quite common in leukemias and sarcomas and sometimes may be the only genetic change seen in these tumours. The Philadelphia

chromosome in chronic myeloid leukaemia is a good example of translocation. Its characterised by the movement of Abelson proto-oncogene (ABL) which is normally on chromosome 9 next to the breakpoint cluster region (BCR gene) on chromosome 22 [t (9;22)(q34;q11)] (Kufe *et al.,* 2003).

1.372 NUMERICAL CHROMOSOMAL ABERRATION

1.3721 ANEUPLOIDY

Aneuploidy is characterised by gain or loss of individual chromosomes from the normal diploid set of 46. Monosomy (i.e. loss of a chromosome) is rare in humans and the vast majority of them abort spontaneously. Most cases of aneuploidy are due to non-dysjunction during mitosis or meiosis. It has been shown that aneuploidy is the most prevalent genetic change recorded among over 20,000 solid tumours analyzed so far (Mitelman 1994). Whether aneuploidy is the cause or consequence of cancer it has been the subject of a long standing debate. There is increasing evidence accumulating in favour of aneuploidy as a discrete chromosome mutation event that contributes to malignant transformation and tumour progression. Studies have shown that the genetic instability of cancer cells is due to aneuploidy and that aneuploidy is a transformation-related event, because it precedes malignant transformation (D.A. Eastmond 1989; Duesberg P 2000; Li R 2000). Aneuploidy has been shown to be a dynamic chromosome mutation event which is associated subsequently with increased rate of chromosomal instability. It has been shown that the rate of subsequent chromosomal aberration is proportional to the degree of pre-existing aneuploidy (Duesberg *et al.,* 1998).

1.3722 MECHANISMS LEADING TO ANEUPLOIDY

The two most important mechanisms leading to aneuploidy are chromosome non-dysjunction and chromosome loss. Other mechanisms that can lead to aneuploidy include non-conjunction, defective centromere division or extra replication of chromosome (Kirsch-Volders *et al.,* 2002). Mitosis is an important process of the cell cycle which is characterised by irreversible segregation of sister chromatids to the daughter cells. Defects in the mitotic-spindle checkpoint and sister-chromatid separation pathways which maintain the fidelity of the process can lead to chromosomal instability and aneuploidy. It is important to know the basic biology of the mitotic-spindle checkpoint and its relationship to chromosome segregation to understand the different mechanisms by which aneuploidy can arise.

During the pro-metaphase the nuclear membrane breaks down and the centrosomes move to the opposite poles. The mitotic spindles are organised between the two centrosomes. The centrosomes are the major microtubule organizing centres (MTOC) and are composed of the centrioles and its surrounding proteinacious matrix such as gamma tubulins and centrosomins A and B (Fukasawa 2005). The slow growing ends of the microtubule (minus end) are anchored in the gamma tubulin and the fast growing ends (plus end) project to find and associate with the kinetochore and are stabilised by this interaction. The spindle checkpoint ensures that all replicated chromatids are properly connected to opposing spindle poles before separation (anaphase) can begin.

Normally anaphase is inhibited by an anaphase inhibiting protein called securin. APC/C (a large Ubiquitin-Protein Ligase) which is the major compoment of the check point triggers anaphase by degradation of securin. APC/C is in turn under the inhibitory control

of MAD2 and BUB3 proteins (Stewart *et al.,* 2003). The BUB and MAD proteins are found to be localised in the unattached kinetochores in the pro-metaphase. Mad2 inhibits the APC activity through direct binding to Cdc20 in the APC complex. MAD2-independent mechanism that involves BUBR1 and BUB3 has also been reported. When all kinetochores of the entire sister chromatids are attached to microtubules, the inhibitory signal is inactivated, and the APC couples ubiquitin chains to its targets.

The targets of the APC/C include the protein securin that hold sister chromatids together and mitotic cyclins (Cyclin–B the mitotic partner of Cdk1) that blocks the onset of anaphase. The sister chromatids are usually joined together at the centromeres and along the chromatid arms by a protein called cohesin. Cohesin is normally disrupted by separin which is under the inhibitory control of securin. Destruction of securin by APC/C activates separin which in turn destroys cohesins resulting in the separation of the two sister chromatids. The mitotic cyclins are destroyed, activating the motors that pull the chromatids to the poles of the spindle (Stewart *et al.,* 2003; Kops *et al.,* 2005).

Mis-segregation of chromosomes may occur as a consequence of a number of problems associated with the separation of sister chromatids during anaphase (figure 1.8). Defects in sister chromatid cohesion, resolution, and separation could all contribute to the genesis of aneuploidy. Failure of the pre-anaphase checkpoint can lead to production of somatic

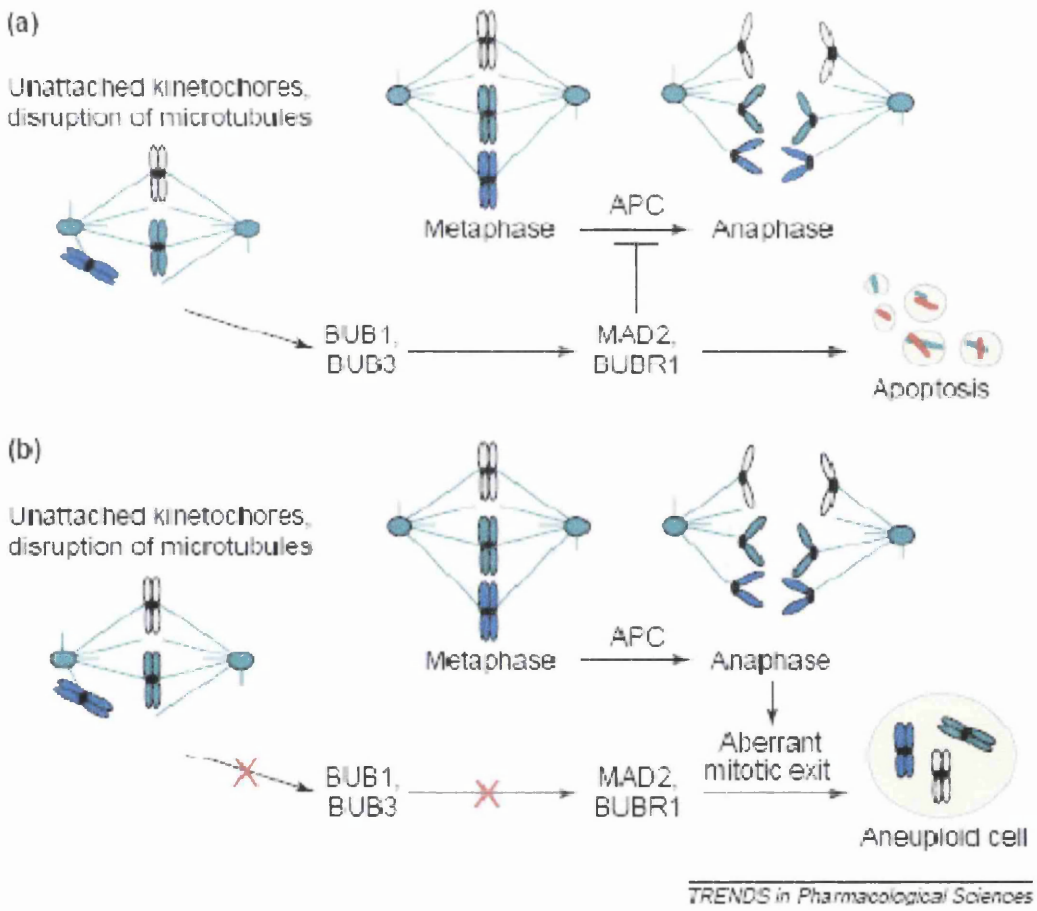


FIGURE 1.8 Mitotic spindle checkpoint can be activated by improper chromosome alignment and disruption of microtubules and unattached kinetochores. (a) intact spindle checkpoint signalling induces metaphase arrest through inhibition of the anaphase-promoting complex (APC) or apoptosis. (b) defective spindle-checkpoint function that results from either loss of BUB1- and BUB-3-dependant signalling or abrogation of MAD2, bubr1-mediated inhibition of the APC can lead to aberrant exit from mitosis and, in the absence of a functional g1-s checkpoint, generate aneuploidy cells (adapted from from Stewart *et al*, trends in pharmacological sciences)

cell populations with abnormal chromosome numbers (Cimini *et al.,* 2005). Other factors which can lead to aneuploidy include abnormal function and number of centrioles leading to multipolar mitoses, kinetochore defects leading to chromosome loss and mitotic slippage resulting from inhibition of mitosis (Fenech 2002).

1.3723 POLYPLOIDY

Polyploidy is the presence of whole sets of chromosomes in excess of the normal number (multiple of the normal 2N). There are various ways in which polyploidy can arise. Errors of fertilization in which a single haploid egg is simultaneously fertilized by two haploid sperm (dispermy) (resulting in three sets of chromosomes, or *triploidy*) or due to errors in the early mitosis or meiosis due to which chromosomes duplicate but cytokinesis (separation of the cytoplasm) fails to occur. Cancer cells frequently show polyploidy.

1.4 PURPOSE OF THE PRESENT STUDY

The strong association of Barrett's oesophagus with oesophageal adenocarcinoma, the incidence of which is rapidly rising makes it a very significant clinical problem. Despite an increase in our knowledge regarding BE the process whereby metaplasia progresses from dysplasia to adenocarcinoma are not completely understood.

Although the progression of BE to OADC occurs in a stepwise manner it is almost impossible to recognize these changes clinically and the only way of monitoring these patients is by periodic surveillance. Surveillance involves endoscopy at regular intervals with four quadrant biopsies being taken at 2cm intervals throughout the length of Barrett's. Owing to the difficulty in detecting patients who progress to adenocarcinoma

the treatment of BE has been controversial and inconsistent. Histology (grade of dysplasia) and patient's symptoms are unreliable parameters to make decisions regarding their management. Almost 20% of the general population suffers from GERD of which almost 10% of these will have BE. Screening all patients with GERD with endoscopy to detect BE would be extremely expensive. We know that inspite of the increase in incidence of OADC the overall five year survival (around 7%) has not improved dramatically. This is mostly because lymphatic vessels (small thin channels similar to blood vessels that do not carry blood, but collect and carry tissue fluid (called lymph) from the body to ultimately drain back into the blood stream) are found in the most superficial aspect of oesophageal mucosa, the involvement of which leads to early lymph node metastasis. Obviously detection of the disease at an early stage would drastically improve the five year survival and cure rates. Current evidence shows that only surgery can eliminate the disease and none of the other treatment modalities have been shown to have any appreciable effect on the progression of dysplasia to OADC. Clearly the best treatment option would be to perform surgery in patients in whom we could predict reliably the progression to OADC. The question which still remains unanswered is which subset of patients should be followed in order to detect OADC at an early and potentially curable stage? The current recommendations of surveillance are imperfect, inefficient and expensive. This is because the guidelines for surveillance are based entirely on the grade of dysplasia which although is the gold standard at the moment, is not a very reliable indicator. Surveillance has not been proved to be cost effective and subjects patients to regular and prolonged discomfort of going through the very unpleasant procedure of endoscopy.

Dysplasia is not a reliable indicator of progression to OADC due to several reasons. Firstly there is considerable inter and intra–observer variation on the interpretation of different grades of dysplasia. Studies comparing the interpretation of eight different pathologists showed that there was an 86% inter-observer agreement for high grade dysplasia and intramucosal-adenocarcinoma while the agreement was 58% and 75% for indefinite dysplasia and low grade dysplasia respectively. This interpretation is further confounded by the presence of oesophageal inflammation (Reid *et al.,* 1988; Kim *et al.,* 1997). The problem of accepting dysplasia as a marker for malignancy is that the natural history of dysplasia is not well defined. It is still not known how fast and how frequently cancer develops in patients with high grade dysplasia (Spechler 2001). Some studies reveal that cancer could develop from high grade dysplasia in a period of 2 to 46 months from the time of diagnosis (Levine 1997) while other studies reveal that high grade dysplasia can persist for years without any progression (Lee 1985; Hameeteman *et al.,* 1989).

The lack of reliability of dysplasia as a marker has lead investigators to seek for more reliable alternative biomarkers. A number of biological events (involving cell proliferation and differentiation) occur during the process of progression to adenocarcinoma. Genetic and molecular changes are the initial events in carcinogenesis. These genetic and molecular signatures could facilitate early detection by high-throughput technologies. Biological markers associated with these events when used alone or in conjunction with histology may help in predicting the progression to carcinoma in these patients. Some of these molecular events may not only help in supplementing and standardizing the diagnosis and progression of dysplasia but may also

serve as targets for therapeutic interventions. The pursuit for biomarker is based on the fact that the onset of disease occurs long before the stage when it can be recognised and identified by pathological means. Hence the usefulness of the biomarkers lies in its ability to provide early indication of disease or progression of the disease. Biomarkers should be easy to detect and measurable across populations. They should enable early detection, identification of high risk individuals and aid in monitoring recurrence. An ideal marker would be one which has low prevalence in non-dysplastic cases and increases with the grade of dysplasia. Theoretically, they could provide the opportunity to intervene during the stage of natural progression of dysplasia to cancer, to cause inhibition, regression, or even elimination of the disease. To be effective and reliable the biomarker should be found in highest frequency in patients with high grade dysplasia and OADC. A large number of biomarkers have been investigated with regards to progression of BE. Some of these include secretory factors, cell cycle regulating factors, adhesion molecules, chromosomal abnormalities, activation of oncogenes (CDKN2/p16, c-src, c-jun, c-Ha-ras, c-erb-B), loss of tumour-suppressor genes (p53), abnormalities in growth regulatory factors (epidermal growth factor, transforming growth factor-alpha) and cell proliferation markers (PCNA) (Fitzgerald *et al.,* 2001; Spechler SJ 2001). Of the numerous biomarkers that have been described the ploidy status and p53 and p16 gene abnormalities are the ones which are most important and intensively documented. Immunohistochemical staining for p53 has been regarded as a marker for malignant progression in Barrett's oesophagus. Not only have several studies shown a poor correlation between positive staining and p53 gene mutations but also frequent accumulation of p53 protein in normal

proliferating epithelium. Mutation of p53 gene or LOH of 17p have been found to be more reliable markers.

The **purpose of this study** was to:

- Understand the genetic changes in the early stages of BE with a view to identify a biomarker which could reliably predict the progression of BE to OADC.
- To develop an in-vitro model of Barrett's oesophagus
- To evaluate the role of bile and acids in the pathogenesis of BE.

1.41 EVALUATION OF EARLY CYTOGENETIC MARKER

A proper understanding of molecular events which trigger the neoplastic process is required in order to potentially identify early markers of malignant transformation which could help in developing treatment and prevention strategies. In order to better understand this process a variety of molecular studies have been carried out in recent years on OADC and BE. Studies have shown that patients with OADC and BE frequently have cells which are aneuploid. Aneuploidy has been detected by various means including DNA flow cytometry, DNA image cytometry, CGH, karyotyping and FISH. Almost 79% to 100% of the patients with OADC have multiple aneuploid populations (McKinley *et al.,* 1987; Reid *et al.,* 1987; Rabinovitch *et al.,* 1989; Krishnadath *et al.,* 1995). Although there are a large number of studies on cytogenetic changes in OADC the number of studies on cytogenetic changes in Barrett's oesophagus is limited. A number of chromosomal aberrations have been described in patients with BE. Persons *et al* showed amplification of multiple chromosomes (6, 7, 11, 12) in patients with BE using fluorescence in-situ hybridization (FISH) with chromosome-specific centromere DNA

probes (Persons *et al.,* 1998). Rabinovitch *et al* using flow cytometry demonstrated aneuploidy in the oesophagus of 14 patients with Barrett's adenocarcinoma (Rabinovitch *et al.,* 1989). Multiple (2 to 14) populations of aneuploid cells were seen in many of the cases. This shows that neoplastic progression in Barrett's oesophagus is associated with a process of genomic instability which leads to evolution of multiple aneuploid populations, with the ultimate development of a clone of cells capable of malignant invasion. Detection of multiple aneuploid populations of cells in Barrett's oesophagus may therefore indicate a high risk of cancer. Aneuploidy has been seen in early stages of BE and the prevalence of aneuploidy has been known to increase with the progression of dysplasia. This could range from 4-6% in early stages to 63-86% in late stages with high grade dysplasia and invasive carcinoma (Reid 1991).

Current data on chromosomal aberrations in BE shows a wide variety of changes, none of which are consistent or reliable in risk assessment. Studies in our own laboratory using comparative genomic hybridization (CGH) on samples from BE revealed widespread chromosome instability in patients with HGD with no significant chromosome changes in patients with LGD (Croft *et al.,* 2002). Amplification of chromosome 4 and 8 were the predominant changes seen in the patients with HGD. CGH is a global assay which can use a small amount of DNA in a single experiment to provide detailed information on gains and losses of genetic material in a tumour. Although CGH is an effective screening tool, it cannot detect aberrations if they are not found in a significantly high proportion in the given tissue. Hence the sensitivity for small amplifications and deletions is poor. FISH on the other hand analyses a specific region of the DNA in the cell. FISH enables more targeted investigations of certain areas of the genome and can detect changes when

found in small magnitude unlike CGH. FISH has become the standard approach for commonly known micro-deletions and micro-duplications.

Our aim was to specifically investigate and compare the numerical aberrations of chromosomes 4 and 8 in patients with BE with various grades of dysplasia using the FISH technique. The specimens were to be obtained from patients undergoing endoscopic biopsy who were already diagnosed with BE. The biopsy samples were to be cultured for a short-term in order to obtain a greater yield of the cells. Theoretically the short-term culture would provide us with actively multiplying cells which could then be arrested in metaphase. The metaphase samples could then be used for karyotyping studies. Whole chromosome probes could be used to detect the karyotypic changes and structural abnormalities of the chromosomes. The short-term cultures could also provide us with interphase cells for cytogenetic analysis with centromeric FISH probes which could potentially detect important numerical chromosomal abnormalities. The advantage of primary culture would be its direct comparibility as they would contain the same combination of cell populations that are also present in the tissue and not sufficient time would have elapsed for selection mechanisms to have taken effect.

1.42 DEVELOPMENT OF IN-VITRO MODEL OF BARRETT'S OESOPHAGUS

One of the purposes of this study was to develop in-vitro model of Barrett's oesophagus. This was to be achieved by long term culture of BE cells obtained from patients undergoing routine endoscopy. Currently there is lack of suitable in-vitro model to study directly the physiology of human oesophageal disease. There are very few investigators who have successfully cultured Barrett's epithelium. Research into molecular and genetic

mechanisms underlying Barrett's oesophagus would be greatly advanced by in- vitro model of Barrett's cells. The development of novel **cell culture** models of Barrett's oesophagus could provide important research tools with which to investigate relevant cellular alterations that may be pertinent to the human disease. Availability of human oesophageal cell culture model could serve as an excellent tool to understand the pathogenesis of human lesions in-vivo, particularly the neoplastic phenomenon. Barrett's cell culture, both long-term and short-term would be an excellent model to study the metaplasia-dysplasia-cancer sequence. Apart from improving our knowledge regarding neoplastic transformation it could also be used to study the effects of carcinogens in-vitro. In addition, such models can serve as the basis for the preclinical testing of new therapeutic strategies. Cell culture model could also be used to obtain sufficient material for a variety of molecular biology, cytogenetic and biochemical and physiological studies. The advantage of in-vitro cell culture is that they allow investigators to study a single effect of a substance or an action in isolation. They also offer high sensitivity without interference from other biological phenomena, such as hormones or immune responses. They can also generate results at faster and far lower cost than methods using live animals. In-spite of all the progress in our knowledge regarding BE the exact aetiology of BE is still unknown. Various factors have been implicated in the development of BE including acid and bile. The cell culture model could be used to study the role of acid and bile which are the most important components implicated in the pathogenesis of the BE and its progression to oesophageal adenocarcinoma. In-vitro studies would give an idea about the cytotoxic and genotoxic effect of these substances. Using natural human cells would make extrapolation of the results easier. It could also be

used as an in-vitro model to study the effects of various carcinogens like polyaromatic hydrocarbons and nitrosamines. Long-term oesophageal organ culture would be an excellent system to study the phenomenon of promotion. This could be achieved by continuous and intermittent exposure of the cells to suspected carcinogens and procarcinogens.

1.43 EVALUATION OF ROLE OF BILE AND ACIDS

Although the exact factors involved in the development of BE are obscure there is ample evidence to prove the strong association between chronic DGER and BE. Various components of the refluxate including gastric acid, bile acids and pancreatic enzymes may be involved in the development of oesophageal injury. Bile acids and gastric acid are the major components of the refluxate which have been implicated in the pathogenesis of BE. To develop better and effective management strategies it is very important to understand the basic factors and the mechanism underlying the development of BE. Studies have shown that patients with BE have more bile reflux compared to patients with uncomplicated GERD. Most of these studies measuring bile reflux are based on Bilitec which is semi-quantitative at best and unreliable in quantifying reflux. With increasing studies showing elevated concentration of bile in patients with BE, the question that remains unanswered is what is the profile of bile acids seen in these patients and in what concentration are they found? Very few studies have actually measured the concentration of different bile acids in these patients. Nehra *et al.,* showed that patients with BE had predominantly taurocholic and glycocholic acids with significant concentration of DCA and TDCA in their bile reflux (Nehra *et al.,* 1999). Most patients had reflux of bile acids in concentrations greater than 200 μmol. Iftikhar *et al.,* found

that patients with BE had bile reflux which was comprised predominantly of GCA and TCA and significant amounts of Taurochenodeoxycholic acid (Iftikhar *et al.,* 1993). The concentration of various bile acids in their study ranged from 20 μmol – 2000 μmol.

Having shown that there is increased bile reflux in patients with BE the next question that needs answering is are they found in significant concentration to cause oesophageal damage or do they have cytotoxic and genotoxic effect in the concentration that they are found in patients with BE.

Information regarding the effects of various bile acids on oesophageal cell lines in-vitro is lacking. Studies performed so far have evaluated the effect of bile acids on colonic mucosa, gastric mucosa or hepatocytes. Bachir *et al* evaluated the effects of high concentration of bile acids (1-3 mmol per litre) on oesophageal organ culture (Bachir *et al.,* 1982). They demonstrated their cytotoxic effects on organ culture at concentrations greater than 2 mmols. There are hardly any studies demonstrating the effects of physiological concentrations of various bile acids on oesophageal cell lines. None of the studies so far have evaluated the genotoxic and cytotoxic effects of various bile acids seen in patients with BE. Evaluation of their cytotoxic and genotoxic potential at various concentrations would give us an idea about their significance and role in the pathogenesis in patients with Barrett's oesophagus. Our study aimed at evaluation of the cytotoxic and genotoxic effects of physiological concentrations of various bile acids seen in patients with BE. The in-vitro studies were to be conducted using oesophageal cell culture model. Long-term primary culture of Barrett's epithelial cells would be an ideal model for this purpose. This would more closely resemble the in- vivo situation with regard to cell type and physiology and biological behaviour. This would make interpretation of results easier

and more readily comparable to the in- vivo situation. The samples for the study were to be obtained from patients with BE undergoing routine endoscopy. Failing this an alternative model of OE33 cells derived from Barrett's oesophageal carcinoma cells was to be used to evaluate the cytotoxic and genotoxic effects of the bile acids. The cytotoxic and genotoxic effects of bile acids were to be evaluated with the in-vitro micronucleus assay which is a validated multi-end point assay. The in-vitro MN assay can simultaneously detect mitotic delay, apoptosis and chromosomal aberrations (both structural and numerical). In the past studies have shown that the bile acids have varying effects depending on the pH and their ionisation state. Conjugated bile acids have a pKa of about 2 and are hence freely soluble at an acidic pH whereas they tend to be precipitated at an alkaline pH. Un-conjugated bile acids on the other-hand have pKa close to 7 and are hence soluble at neutral pH and precipitate in solution at acidic pH. Owing to this, conjugated bile acids can be most harmful at acidic pH while un-conjugated bile acids can be more harmful at neutral pH. In order to evaluate the effects of bile acids in varying pH conditions the experiments were to be conducted in both acidic and neutral pH conditions.

Although demonstration of tumour induction in vivo would be a definite proof for tumour promoting or carcinogenic activity of an agent it may not be always possible. Animal experiments can be expensive and time consuming. A number of studies have shown the tumour inducing capacity of bile acids in animals but extrapolation of these results to human situation is difficult. Owing to this our in-vitro model would be a good tool to demonstrate the effect of various agents possibly involved in the development of BE.

CHAPTER TWO

BARRETT'S CELL CULTURE MODEL

AND INTERPHASE FISH STUDIES

2.1 REVIEW OF OESOPHAGEAL / BARRETT'S TISSUE CULTURE

Tissue culture has been performed since the beginning of the twentieth century (Harrison 1907). The first solid tumour-derived cell line, termed HeLa, was established by George Gey at the Johns Hopkins Medical School in 1951 from a 31-year-old mother of four, Henrietta Lacks (Gey *et al.,* 1951). Since the classical work of Hayflick and Morrhead (Hayflick *et al.,* 1961). There has been an explosive expansion in tissue culture technology. Browning and Trier were the first to culture adult human intestinal mucosal biopsies in vitro for a twenty four hour period (Browning *et al.,* 1969). A number of workers have used the technique described by Browning and Trier with minor modifications which is based on supporting the tissue in contact with, but not submerged, in suitable culture medium, in an oxygen enriched atmosphere. Browning and Trier placed the mucosal specimen on a sterile steel wire grid placed on a central well of a culture dish as shown in the diagram (figure 2.1). The central well was filled with culture medium so as to just touch the under surface of the mucosa. The principles of explant tissue culture still remain unchanged. Culture of alimentary tract mucosa has proved to be very difficult compared to cells and tissues from most other organ systems (Trier 1976). Availability of human oesophageal cell culture could serve as an excellent model to understand the pathogenesis of human lesions in-vivo, particularly the neoplastic

phenomenon. It could also be used as an in vitro model to study the effects of various carcinogens like polyaromatic hydrocarbons, nitrosamines, bile acids and stomach acid. Long-term oesophageal organ culture would be an excellent system to study the phenomenon of promotion. This could be achieved by continuous and intermittent exposure of the cells to suspected carcinogens and pro-carcinogens. Availability of Barrett's cell culture, both long term and short term, would be an excellent model to study the metaplasia-dysplasia-cancer sequence, effects of carcinogens and neoplastic transformation, to test potential therapies for BE and to acquire sufficient material for a variety of molecular biology, cytogenetic and biochemical and physiological studies.

A review of the literature shows that there are very few reports of methods for in vitro culture of Barrett's epithelial cells with varying degrees of success (table 2.1). A long term model of non transformed primary cell line is still lacking for Barrett's oesophagus.

The first attempts to culture human oesophagus was made by Syverton and Mclaren in 1957. Syverton and Mclaren reported the establishment of an oesophageal epithelial strain from a one day old infant with tracheobronchial fistula (Syverton *et al.,* 1957). Aydeolotte was successful at growing animal embryonic oesophageal epithelium for more than two weeks (Aydelotte 1963).

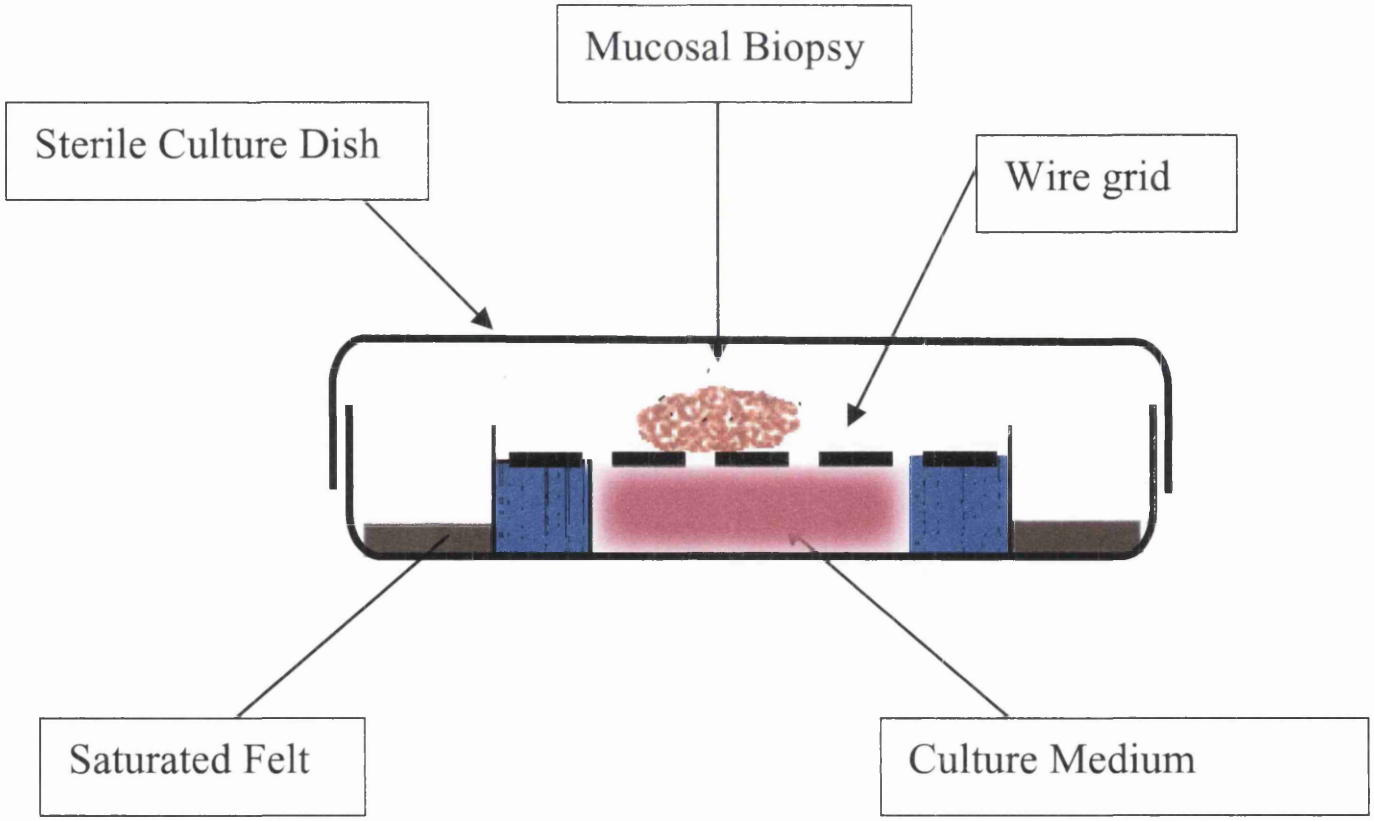


FIGURE 2.1 Diagrammatic representation of organ culture used by Browning and Trier (Adapted from Howdle 1984)

In 1975 Rosztoczy *et al.,* cultured normal human fetal, and adult oesophageal epithelium for up to seven days. His organ culture supported the replication of influenza virus for about four days (Rosztoczy *et al.,* 1975). Hilman *et al.,* were the first to maintain normal oesophageal tissue in organ culture for a long period of time (Hillman *et al.,* 1980). They were able to maintain oesophageal explants obtained from autopsy specimens or surgically resected specimens for a period of 6-16 weeks. Hilman *et al.,* used explants of $1cm^3$ from various segments of oesophagus. The explants were grown in 3-5ml of culture medium (CMRL 1066) which was supplemented with insulin, hydrocortisone and 5% fetal calf serum. The culture dishes were placed on a rocker platform to make the medium flow intermittently over the epithelial surface. Although Hilman *et al.,* managed to maintain explants for more than a few weeks they never grew cells in monolayers (Hillman *et al.,* 1980).

Hilman *et al.,* attempted cultures from 18 subjects of which seven were lost due to infection and the rest were maintained in culture for about 6- 16 weeks. Katayama *et al.,* used tissue samples from non-tumorous part of surgically resected specimen (Katayama *et al.,* 1984). They disaggregated the cells by means of tryptic digestion and grew them in RITC80-7 supplemented with 10% fetal bovine serum (FBS). They established primary cultures from 20 donors and were able to passage them three to five times. Additional supplements to their media included epidermal growth factor (EGF), insulin, transferrin, FN and BSA.

TABLE 2.1

Showing list of studies conducted on oesophageal cell culture

AUTHORS	YEAR	BARRETT'S/ NORMAL	SOURCE OF TISSUE	TECHNIQUE USED	
				Explant*	Enzymatic
Hilman *et al.,*	1980	Normal	Autopsy/ Surgically Resected	++++	
Zboralske *et al.,*	1984	Normal	Autopsy & Heart/Lung Transplant	++++	
Katayama *et al.,*	1984	Normal	Surgically Resected		++++
Mothersill *et al.,*	1989	Normal	Surgically Resected	++++	
Resau *et al.,*	1990	Normal	Autopsy	+++ +	++++
Garewal *et al.,*	1992	Barrett's	Endoscopic biopsies	++++	
Washington *et al.,*	1994	Barrett's	Endoscopic biopsies	++++	++++
Khan *et al.,*	1997	Barrett's	Endoscopic biopsies	++++	
Wessels *et al.,*	1998	Barrett's	Endoscopic biopsies	++++	++++
Wessels *et al.,*	2003	Barrett's	Endoscopic biopsies	Telomerase Reverse Transcriptase	

* Most authors have used the explant method, and in those studies where both methods have been used, it is mostly the explant method which has been employed predominantly.

Zboralske *et al.,* used tissue samples from heart/lung donors and autopsy specimens. They used explants of 1.5 ×1.5 mm^3 (Zboralske *et al.,* 1984). They used a unique method to fix the explant to the culture dish. A drop of chick plasma with fibrinogen (18mg /ml) was used and one to two drops of bovine thrombin (100U/ml) to anchor the explant to the plastic Petri-dish. After clot fixation of the explant 1.5ml of the culture medium was added. They used Eagle's minimum essential media supplemented with penicillin (200U/ml) /streptomycin (100μg/ml), 10% fetal bovine serum and dexamethasone

1μg/ml. Of the eleven different samples used they were able to obtain cultures from six patients and a maximum of two serial passages of the cells.

Mothersill *et al.,* in 1989 described a technique for short-term culture of human oesophageal epithelial cells (Mothersill *et al.,* 1989). They used a combination of explant and enzymatic methods to grow normal oesophageal mucosa obtained from surgically resected specimen. In their attempts to optimise medium for the culture of oesophageal cells, Mothersill *et al.,* found that a medium rich in amino acid supplements promoted healthy fibrobast free growth. They found that supplementation of these amino acid rich mediums with 10% serum (horse, newborn or foetal calf serum), insulin, hydrocortisone, insulin and EGF was ideal for the growth of oesophageal cells. The techniques used by Mothersill *et al.,* enabled them to grow the cells for a maximum of four weeks.

Resau *et al.,* obtained oesophageal cells from autopsy specimens (Resau *et al.,* 1990). They used both explant and enzymatic method to initiate their cultures. They compared the effect of serum free keratinocyte growth medium and serum supplemented CMRL 1066 on the growth of cells in culture. They found that the viability and differentiation of the epithelial cells was better in KGM compared to serum supplemented media.

The first report of culture of Barrett's oesophagus was made by Garewal *et al.,* in 1992. They were the first to obtain monolayer cultures from Barrett's cells (Garewal *et al.,* 1992). They used the explant method to culture Barrett's specimens obtained from endoscopic biopsies. The tissue was finely minced and were grown in T25 plastic collagen coated culture flasks. They used the M-19 medium with bovine pituitary extract supplemented with 20% foetal bovine serum. By using this technique they were able to subculture the cells by about six times on an average and most cells showed degenerative

changes by passage 8-10. None of their cells were immortal. Garewal et al had 60-70% success, with others lost either due to inability to grow, fibroblast overgrowth or infection.

Washington *et al.,* obtained tissue from surgically resected specimens (Washington *et al.,* 1994). They used both explant and enzymatic method (using trypsin) to initiate their cultures. Their culture media consisted of MCDB-153 with 5% calf serum and supplemented with hydrocortisone, bovine pituitary extract, penicillin, streptomycin and amphotercin. They were able to subculture for a maximum of six to eight times with tissues being maintained in vitro for an average of three months. Of the 12 cultures that were started four were lost to infection and one failed to grow. Washington et al noticed that cells grown on type IV collagen grew more slowly than cultures grown on uncoated plastic, and could be sub-cultured fewer times.

Khan *et al.,* used the explant technique to culture Barrett's samples from endoscopic biopsies(Khan *et al.,* 1997). They used DMEM and HAM F12 in the ratio 3:1 supplemented with 10% fetal bovine serum to initiate their cultures (table2.2). This medium was later replaced by keratinocyte serum free medium for culture propagation. Of the sixteen samples five were lost due to infection. Eleven samples could be sub-cultured several times (most of them 8-10 times and a maximum of 20). According to Khan et al the use of keratinocyte serum free medium not only eliminates the fibroblast overgrowth, but also prevents serum induced terminal differentiation of the epithelial cells.

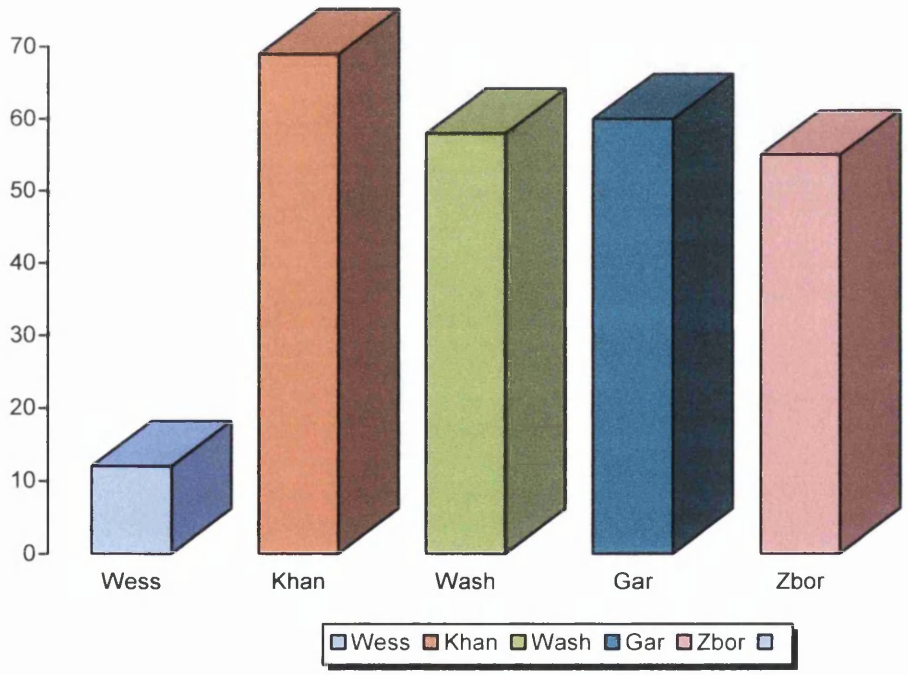
TABLE 2.2 Table showing the comparison of the composition of cell culture medium used in different studies.

	ZBORALSKE ET AL	MOTHERSILL ET AL	RESAU ET AL	GAREWAL ET AL	WASHINGTON ET AL	KHAN ET AL	WESSELS ET AL
BASIC MEDIUM	EAGLES MEM	MEM	KGM	M-19	MCDB-153	DMEM+ HAM F1*	MCDB-153
SERUM	10%FBS	20% FCS		20%FBS	5%FCS	10% FBS	5% FBS
INSULIN		10mIU/ml	5µg/ml			5µg/ml	5µg/ml
HYDROCORTISONE		1µg/ml			.4µg/ml	1.2mg/ml	.4µg/ml
DEXAMETHASONE	10µg/ml						
EGF					20ng/ml	50ng/ml	20ng/ml
CHOLERA TOXIN					10^{-10} mol/ml	10^{-10} mol/ml	10^{-10} mol/ml
TRANSFERRIN			5µg/ml			5µg/ml	5µg/ml
BOVINE PITUIT EXTRACT			+++	+++	140µg/ml	50mg/ml	140µg/ml
ADENINE					1.8×10^{-4}M	24mg/mlL	
GLUTAMINE		+++					4mmol/ml
SELENIUM			5ng/ml				5ng/ml
PENICILLIN		20U/ml	+++		100U/ml	200U/ml	100U/ml
STREPTOMYCIN		20U/ml	++ +		100µg/ml	200µg/ml	100µg/ml
GENTAMICIN		4u/ml				20µg/ml	
AMPHOTERCIN-B		1µg/m/	0.50µg/ml		0.25µg/ml		0.25µg/ml

TABLE 2.3
Showing the results of oesophageal cell culture from various studies

	ZBORALSKE ET AL	GAREWAL ET AL	WASHINGTON ET AL	KHAN ET AL	WESSELS ET AL
No of Samples	11	-	12	16	33
Infection in %	5	-	4	5	-
No of passages	2	8-10	6-8	8-10	20
No Positive cultures	6	60-70%	7	11	4
Immortal lines	None	None	None	None	None
%age of success	55%	60-70%	58%	69%	12%

Graph showing the success rate of various studies for oesophageal cell culture



Wessels *et al* used both enzymatic and explant technique for initiation of culture of their samples obtained from endoscopic biopsies(Palanca-Wessels *et al.,* 1998). Like Washington *et al* they used MCDB 153 and 5%fetal bovine serum and other ingredients to initiate and propagate their cultures

Attempts were made to initiate 39 primary cultures from thirty three different patients. They managed to obtain long term culture from four different samples which could be sub-cultured up to 20 times (see table 2.3). The rest of the samples failed to grow or were lost to infection.

Recent studies have shown that cell culture life span is related to chromosomal telomere length, and cellular senescence results from progressive telomere shortening (Bodnar *et al.,* 1998). Based on this findings Wessels *et al.,* (Palanca-Wessels *et al.,* 2003) transduced primary cultures of Barrett's esophageal epithelium with a retrovirus containing hTERT (human catalytic subunit of telomerase reverse transcriptase). The reactivation of telomerase enhanced the genomic stability and increased the life span of the Barrett's cells.

2.2 FISH

2.21 INTRODUCTION

FISH has become a powerful molecular tool for detecting cytogenetic and molecular genetic alterations, and is widely applied in various fields of study (Ekong *et al.,* 1998). Since its inception about 30 years ago it has evolved into a highly effective technique which can detect multiple unique sequences simultaneously with different colours (Andreeff M 1999). In situ hybridization was first described by Pardue and Gall in 1969. They used radioactive labeled repetitive DNA probes in their studies (Pardue *et al.,* 1969). This technique had obvious limitations due to its heavy reliability on radioisotopes and autoradiography for the visualization of the hybridized gene probe.

The current technique of FISH using non-radioactively labeled probes was developed Pinkel *et al* (Pinkel *et al.,* 1986). Genomic *in-situ* hybridization can be defined as a method involving hybridization of labeled total genomic DNA, used as a probe, to

spread or sectioned denatured chromosome preparation *in-situ*, followed by detection and visualization of the sites of hybridization (Clark 2002).

2.22 PRINCIPLES OF FISH

The nucleic acid in the target cells or chromosomes is denatured i.e. made single stranded (e.g. by heating). Once the DNA is denatured it is incubated with labelled single stranded probe nucleic acid molecule under conditions that permit binding of molecules that have complementary base pair sequences. The probe is then detected either by direct or indirect method (see figure 2.2). In the direct method labels such as flurochromes [FITC, Texas Red or Cyanine dyes (Bauman *et al.,* 1980) or enzymes (Renz *et al.,* 1984)] are directly coupled to the nucleic acid probe molecule. In the indirect technique the nucleic acid probe is labeled with a hapten (such as biotin or digoxigenin) which after in situ hybridization, is detected by immunocytochemical means (Raap 1998). The haptens can be detected by means of fluorescently labelled molecules which have a very high affinity for the haptens. The signals may be amplified by the use of simulataneous layers of antibodies and antibodies conjugated to fluorescent molecules (Ekong *et al.,* 1998).

In the direct method, apart from being able to visualize the results directly, there is no background staining due to nonspecific binding of the immunocytochemical reagents (Raap AK 1990). After hybridization the numbers, intensities, and spatial distribution of each of the different coloured hybridization signals can be determined (Andreeff M 1999).

2.23 APPLICATIONS OF FISH

FISH can be used for the detection of numerical and structural chromosomal aberration. A variety of FISH probes are available, the choice of which depends on the purpose of the study. FISH probes can detect tandem repeats, dispersed repeats and complete genes in the chromosome. Repetitive α satellite DNA sequences specific

for centromeric region can be used to detect the chromosome copy number in the interphase cells. FISH has been successfully used for the analysis of numerical chromosomal abnormalities in interphase nuclei of various solid tumors (Hopman *et al.,* 1989; Baretton *et al.,* 1994). Interphase FISH enables examination of cells from tissue sections or tumour cell suspension without resorting to tissue culture. It can also be used to analyze stored archival samples ranging in age from a few months to several years. Because of the ease and speed with which it can be performed, interphase FISH has become a well accepted technique in cancer cytogenetics. With efficient hybridization and proper scoring interphase FISH can produce results comparable to metaphase analysis (Eastmond *et al.,* 1995).

Whole chromosome probes which label the entire length of the chromosome can be used to analyze metaphase cells for both numerical and structural chromosomal abnormalities. Whole chromosome probes make it possible to detect translocations, insertions and breakage points (Sharma *et al.,* 2001).

Fish can also be used for karyotyping of cells. Several new techniques like M-FISH (Multiplex fluorescence in situ hybridization), SKY (spectral karyotyping) and colour changing karyotyping have been used to detect complex structural and numerical chromosomal aberrations. Although the conventional G banding is commonly used for karyotyping, it cannot detect minute structural aberrations. SKY/M-FISH can localize all the genes simultaneously and hence can be used for marker chromosome characterisation in cancer and other disease states. The limitations of M-FISH /SKY are that they are expensive, technically demanding and need proliferating material.

Another recent FISH technique is CGH (comparative genomic hybridisation) which involves the hybridisation of equal amounts of test and reference DNA, labelled in two different colours, to a normal metaphase spread. Regions with equi-molar DNA

appear yellow, where as regions with under or over represented DNA appear red or greenish (van Ommen *et al.,* 1995). CGH is a powerful tool which can be used to detect inter and intra-chromosomal imbalances, gene amplifications and for marker chromosome characterization. The limitations of CGH is that it cannot detect balanced aberrations or small imbalances, needs cytogenetic expertise for karyotyping, and small mosaics can be easily missed (Tonnies 2002).

Fibre FISH is another sensitive high resolution technique. It enables inspection specific DNA sequences along a stretched single DNA fibre. The theoretical resolution of fibre fish is almost 1 kb. Thus the applications of FISH are widespread and continuously expanding, with the evolution in the FISH techniques.

2.3 MATERIAL AND METHODS

2.31 ROUTINE LABORATORY PROCEDURES

TISSUE CULTURE EQUIPMENT

Glassware and pipette tips were sterilised in an autoclave (Prior Clave Ltd) at 121°C and 15p.s.i for 30 minutes. Disposable sterile plastic tissue culture flasks, wells, pipettes, syringes, centrifuge tubes, Petri-dishes, cryovials and universals were purchased from Nunc A/S, Life Technologies Ltd. UK, Sterilin Ltd. Staffordshire UK or Alpha. All cell cultures were carried out under sterile conditions in a laminar flow cabinet (Cytomat Safety Cabinet, Medical Air Technologies Ltd, Manchester UK.). All incubations were done in LEEC Mk11 Proportional Temperature Controller (LEEC Ltd Nottingham UK) incubators at 37°C and 5% CO_2.

MEDIA, REAGENTS AND CHEMICALS

Chemicals and media were purchased from the companies mentioned previously with the respective product. Growth media and other culturing solutions prepared in the laboratory were sterilised by autoclaving at 121°C and p.s.i for 30 minutes. Where

reagents were unsuitable for autoclaving, filter sterilisation using 0.2μm filtration discs (Acrodiscs, Gelman Sciences) was employed. Purified water was obtained from a reverse-osmosis water purification system (Millipore).

1. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-Brl)

2. Ham's F-12 Nutrient Mixture (Gibco-Brl)

3. Epigrow / Keratinocyte Serum Free Medium

4. Foetal Bovine Serum-FBS (Gibco-Brl)

5. 0.05% (w/v) Trypsin- 0.02% (w/v) EDTA (Gibco-Brl)

6. Adenine

0.97g of adenine (Sigma) was added to 20ml of distilled water. 10 Molar sodium hydroxide was added dropwise until adenine dissolved or a pH of 9.0 was reached. This 0.36M stock of adenine was aliquoted into 0.1ml volumes into cryovials and stored at -20°C until required. A further working stock of adenine was prepared by diluting a 1.0ml aliquot of 0.36M stock one in twenty in DMEM to give a 0.018M working stock. This 0.018M stock was aliquoted into 1.0ml volumes into cryovials and stored at -20°C until required.

7. Cholera Toxin

1.0mg freeze-dried preparation of Cholera toxin (sigma) was added to 12.0ml of DMEM and aliquoted into 1.0ml volumes and stored at 4°C until required.

8. Transferrin

A 100mg quantity of transferrin (Sigma) was dissolved in 10mls of distilled water to produce a stock of 10mg/ml transferrin. This was aliquoted into 1.0ml volumes into cryovials and stored at -20°C until required.

9. Insulin Stock

100mg if Insulin (Sigma) was dissolved in 10mls of 0.05M hydrochloric acid to produce a stock of 10mg/ml insulin. This was aliquoted into 1.0ml volumes and

stored at -20°C until required. 1.0ml of 10mg/ml insulin was added to 9.0ml DMEM to produce a working stock of 1.0mg/ml. This was aliquoted into 0.5ml volumes into cryovials and stored at -20°C until required.

10. Epidermal Growth Factor Stock

10μg of epidermal growth factor (Sigma) was dissolved in 1.0ml DMEM to produce a stock of 10μg/ml. This was aliquoted into 5μg volumes into small eppendorf tubes and stored at -20°C until required.

11. Hydrocortisone Stock

1.0mg of hydrocortisone (Sigma) was dissolved in 1.0ml absolute ethanol and then added to 19.0ml DMEM to produce a 50μg/ml stock. This was aliquoted into 800μg volume in cryovials and stored at -20°C until required.

12. Bovine Pituitary Extract (Sigma)

13. 1% (v/v) 200mM l-Glutamine (Gibco-Brl)

14. Penicillin-Streptomycin, 10,000 units of penicillin G and 10,000 μg of streptomycin sulfate per ml (Gibco-Brl)

15. Amphotericin B 0.125μg/ml (Gibco-Brl)

16. Sterile scalpel and two pairs of forceps

17. Collagenase (Sigma)

The culture media was freshly prepared and used for no longer than three weeks. The culture medium was stored in the fridge at 4°C. The composition of the medium used for the intiation of primary culture was as follows:

3:1(v/v) mixture of Dulbecco's modification of Eagles medium-DMEM (Gibco-Brl) and Ham F12 nutrient mixture (Gibco-Brl). To this was added

- Serum 10% FBS
- Insulin 5μg/ml

- Hydrocortisone 4μg/ml
- EGF 20ng/ml
- Cholera Toxin 10^{-10} Mol/ml
- Transferrin 5μg/ml
- Bovine Pituitary Extract 140μg/ml
- Adenine $1.8x10^{-4}$ M
- Glutamine 4mmol/ml
- Penicillin
- Streptomycin
- Amphotercin-B

} Varying concentrations used for different experiments

CHEMICALS AND REAGENTS FOR FISH

2 X SSC: 40mls of 20X SSC + 360mls of water

0.4 X SSC with 0.3% NP-40: 2ml of 20 X SSC+ 98ml of water + 300μL of NP-40

2 X SSC with 0.1% NP-40: 10mls of 20 X SSC+ 90ml water + 100μL of NP-40

2.32 TISSUE COLLECTION

Tissue samples were obtained from patients with previously diagnosed Barrett's oesophagus attending endoscopy clinic at Morriston Hospital, Swansea. Ethical approval was obtained from local research ethics committee. All patients gave informed consent to participate in the study. Three to six biopsies were taken from each patient depending on the length of Barrett's segment. For the confirmation of the diagnosis, either an adjacent biopsy was taken or, if a single biopsy was large it was split and sent for histological evaluation. The tissue sample was placed immediately in the transport medium (TCM) and kept at 4° C until further processing. All the specimens were processed on the same day approximately within two hours of procuring the biopsy specimen.

2.33 CELL CULTURE PROCEDURE

The cell culture was initiated using two different techniques, namely the explant technique and the enzymatic technique.

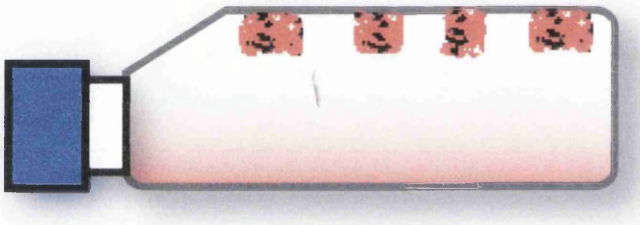
2.331 EXPLANT TECHNIQUE

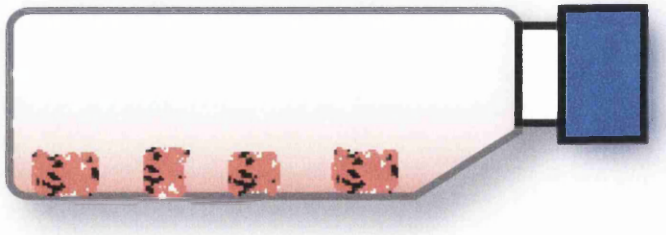
The explant technique used was similar to the one described by previous workers (Garewal *et al.,* 1992, Khan *et al.,* 1997). The biopsy specimens were placed in a sterile Petri-dish, and minced to fragments of approximately 1-2mm^3 in size using a sterile scalpel blade (see fig.2.3). The minced fragments were suspended in 5ml of the growth medium and centrifuged at 1500rpm for eight minutes. The supernatant was discarded and the pellet was washed three times by re-suspending in the growth media and mixing the cells with a pipette, and centrifuging at 1500rpm. Finally the supernatant was discarded and re-suspended in 1ml of the tissue culture medium. This was transferred to 25cm^3 tissue culture flask. The minces were distributed uniformly with the help of a Pasteur pipette on the bottom of the flask. The flasks were then left upside down for an hour. This hanging drop effect enabled the attachment of the minces to the surface of the flask. Later on 0.5ml to 1ml of the tissue culture medium was added to each flask and incubated at 37°C in a humidified atmosphere containing 5% CO_2. On average, three to five flasks containing three to five explants per flask were used. The flasks were left undisturbed for about 3-4 days. Once the pieces were seen to have cells growing from them, the volume of the medium was gradually increased to 5ml over several days.

FIGURE 2.3 PRIMARY EXPLANT TECHNIQUE

TISSUE WAS FINELY CHOPPED WITH A STERILE KNIFE AND WASHED SEVERAL TIMES IN THE CULTURE MEDIUM BY CENTR -IFUGING AT AT 1500 RPM FOR 8 MINUTES

THE FLASKS WERE LEFT UPSIDE DOWN FOR AN HOUR IN ORDER TO AID THE ATTACHMENT OF THE BIOPSY FRAGMENTS TO THE SURFACE OF THE FLASK





THIN FILM OF MEDIUM WAS ADDED TO THE FLASKS, AND THE FLASKS WERE LEFT UNDISTURBED FOR 3-5 DAYS

2.332 ENZYMATIC METHOD

In the enzymatic method, the whole biopsy specimen was placed in a sterile Petri-dish containing 1mg/ml of collagenase III in 1x phosphate buffered saline and incubated at 37°C for about one to two hours. The tissue was gently syringed to dissociate the cells. The resulting suspension was centrifuged at 1500rpm for eight minutes.

The supernatant was discarded and the pellet was re-suspended in culture medium. The cells were counted using a haemocytometer. They were then seeded into tissue culture flask (T25) at a cell density of $2x10^5$cells/cm^2. The flasks were then incubated at 37°C in a humidified atmosphere containing 5% CO_2.

After initiation of the primary cell growth the medium was changed twice a week. When the cells covered more than 50% of the surface of the flask they were split by incubating with 0.25% trypsin with EDTA at 37°C for 5-10 minutes. The resulting suspension was then transferred into a centrifuge tube and centrifuged at 1500rpm for eight minutes. The cells were then plated in the tissue culture flasks at a density of $2x10^5$cells/cm^2. For the subsequent culture of the cells Epigrow, a commercially available serum free medium for epithelial cells was used.

HARVESTING CELLS

Once the cells occupied more than 75% of the surface of the flask they were split with 0.25% trypsin with EDTA. The culture medium was removed by suction and discarded. About 3ml of 0.25% trypsin with EDTA solution was added to the flask. The solution was discarded immediately and another 3ml of Trypsin/EDTA solution was added. The cells were detached completely by shaking the flask several times and about 10 ml of medium was added to the flask. The cells were sub-cultured in a ratio of 1:2. Fresh culture medium was added to each flask to make up the quantity to

10mls. The flasks were gassed with CO_2 and then incubated at 37°c in humidified atmosphere and 5% CO_2.

2.34 SLIDE PREPARATION

The cells were detached with Trypsin EDTA, as described in the previous section. The cells were re-suspended in the culture medium in a concentration of about 1 x 10^5 cells/ml. Aliquots of 50- 100µl were centrifuged in the cytospin (Shandon) for 8 minutes at 200 x g. The quality of the slides was checked under a light microscope. The slides were then air dried for a brief period and then fixed with 90%v/v methanol at -20°C for 10 minutes. 2-4 slides were prepared from each culture. After fixing the cells were air dried.

2.35 INTERPHASE CYTOGENETICS (FISH WITH CEP PROBES)

The slides were prepared as described in the previous section. Fish was performed according to the protocol supplied with the Vysis probes with minor modifications (0.5µl of each CEP DNA probe was useed instead of 1µl).

Vysis CEP probes – Chromosome Enumeration Probes – obtained from Vysis UK (Vysis, Surrey UK). Chromosome enumeration probes (CEP) consist of chromosome-specific tandem repeat DNA sequence (alpha satellite, satellite-I or satellite-III DNA). They are directly labelled with a Vysis fluorophore for detection. Unlabelled blocking DNA is mixed with the DNA sequence to suppress the more highly repetitive sequences that are common to many different chromosomes. To minimise cross – hybridization to other similar repeat sequences on other chromosomes the DNA sequences are hybridizesed under conditions of high stringency. For each test specimen a minimum of 200 nuclei were counted and the number of fluroscent signals per nucleus was recorded. FISH was performed using the following protocol:

Slide preparation:

The slides were washed in 2xSSC for 2 minutes at room temperature. The slides were then dehydrated in a series of ethanol, 2 minutes in each of 70%, 80% and 100% v/v ethanol and air dried.

Probe preparation:

Due to limited number of slides available for each sample, two probes were simultaneously hybridized in each reaction. The probe was prepared by adding the following constituents to a micro-centrifuge tube at room temperature: 3.5µl of CEP hybridization buffer, 0.5µl of each CEP DNA probe, and, to a 5µl volume. The mixture was centrifuged 1-3 seconds, vortexed and re-centrifuged briefly.

Hybridization:

5µl of each probe mix was added to the cytodot. A plastic cover-slip on top of the cytodot and immediately the edges were sealed with rubber cement. The slides were then placed on a hot plate at 75°C for 2 minutes in order to denature the probe. The slides were then incubated at 37°C for 30 minutes. A Coplin jar with a solution of 0.4xSSC/0.3% NP-40 was pre-warmed in a 73°C water bath at least 30 minutes prior to use. After incubation, the cover-slips were removed and the slides were immersed in the pre-warmed solution for 2 minutes. The slides were then washed in 2xSSC/0.1% NP-40 for 5-10 seconds. The slides were dried and 10ul of DAPI was applied to each slide. The slides were viewed after 10 minutes under a fluorescence microscope using a triple band-pass filter, which permits the simultaneous detection of DAPI (white), propidium iodide (orange), Cy-3 (red) and FITC (green). The excitation and emission wave lengths for different colours were as show below:

Colour	Excitation (λ)	Emission(λ)
DAPI (white)	345	455
FITC (green)	494	518
Prop Iodide (orange)	536	617
Cy3 (red)	550	565

2.36 FISH ANALYSIS USING WHOLE CHROMOSOME PROBES

WHOLE CHROMOSOME FISH SOLUTIONS

20 X SSC 3.0M NaCl (17.53g per 100ml)

0.3M Trisodium citrate (8.82g per 100ml)

Formamide (SIGMA)

De-iodinise by stirring for 30 min with 10g of amberlite monobed resin (SIGMA) per 100ml, then filter twice.

Denaturation solution

4ml 20 X SSC

8ml distilled water

28ml formamide

Post-hybridization washing solution

7.5mls 2 X SSC pH 7.0

7.5 ml distilled water

15mls formamide

Washing solution

100ml 20 X SSC

400ml distilled water

250 Tween-20

Blocking reagent

2mg sodium azide

10ml washing solution

500mg Bovine Serum Albumin

Warm to 37°C to dissolve. Store at 4°C for up to 2 weeks.

2.361 METAPHASE PREPARATION

A confluent 50cm^2 flask was split and seeded into a 25cm^2 flask with a cell concentration of about 1-2 x 10^{5}cells/ml in 10 mls of the culture medium. The flasks were gassed and incubated at 37°C for 48hrs to recover from the trypsin treatment. After 48 hrs the medium was renewed and gassed and the cells were treated with 10µl (1µg/ml) colcemid (Life Technologies) and left overnight in the incubator at 37°C. The next day the cells were trypsinised and centrifuged at 1200rpm for 8 minutes. After centrifugation the cell pellet was re-suspended slowly in warm (37°C) KCl (0.56%) incubated for about 10-12 min at 37°C. The cells were then centrifuged at 1200rpm for 8 min. The cell pellets were re-suspended in ice cold fix (3:1, methanol: acetic acid) and centrifuged at 1200rpm for 8 minutes. This procedure was repeated two more times. After the final centrifugation the cells were re-suspended in a small quantity of fresh ice cold fix. Slides immersed in ethanol were cleaned. With a liquipette microtip (Elkay) an aliquot of cells was dropped on to a clean slide from a height. The slide was left to dry at room temperature and then was examined for the presence of metaphases using a light microscope.

2.362 FISH ANALYSIS USING WHOLE CHROMOSOME PROBES

Whole chromosome painting was done using Cambio FISH probes. The protocol used was the one supplied by the manufacturers (Cambio). Whole chromosome probes for all the chromosomes except 9, 21, 22 and Y chromosome were used (owing to lack of probes). The protocol used was as follows:

DAY 1:

Fresh metaphase slides were prepared and air dried. The slides were then fixed for 1 hr in fix (3:1 methanol:acetic acid) and then left to dry. The slides were then dehydrated in ethanol series for 2 min in each of 70%, 80%, 95% and 100% ethanol and left to dry overnight in an incubator at 42°C.

DAY 2:

The slides were fixed in acetone in a Coplin jar for 10 min and then air dried. The paints were warmed to 42°C and mixed well before use. An appropriate amount of paint (10 µl per slide) was denatured by incubation at 65°C in a water bath for 10 minutes. 5 µl of each of two chromosome probes (Cambio) were added to a micro-centrifuge tube (two tubes were prepared each containing two chromosome probes) and was transferred to a 37°C water bath and was incubated for 1 hr.

The slides were then denatured by incubation in a Coplin jar with 70%v/v formamide/2xSSC at a water bath at 65°C for 2 minutes. The slides were then quenched in an ice cold ethanol series (70%, 80%, 95% and 100%v/v) for 2 minutes in each and then left to dry. 10µl of probe was added to each end of the slide (two at each end). Cover slips were placed on each end and sealed with rubber cement. The slides were then incubated overnight at 42°C in a humidified chamber.

DAY 3:

The following Coplin jars were prepared and pre-warmed in a water bath at 42°C:

3 jars with 2xSSC (pH7)

2 jars with post-hybridization wash

9 jars with wash solution

The rubber cement was removed and the coverslips were rinsed in 2xSSC (pH7) at 42°C. The slides were washed by incubating for 5 minutes in each of two jars of

50%formamide/0.5xSSC. The slides were then washed by incubating for 5 minutes at 42°C in 2xSSC.

50µl of Avidin Texas Red (Vector) (diluted 1:500 blocking reagent) was added to each end of a slide and covered with a plastic coverslip. The slides were incubated at 37°C for 20 minutes in a humidified chamber. After incubation the slides were washed three times in wash solution at 42°C for 5 minutes each.

50µl of the following mix was added to each end of the slide:

1.25µl F1 (Detection reagent, Cambio)

500µl blocking reagent

1µl biotin antiavidin (Vector)

The slides were then covered with plastic coverslip and incubated at 37°C for 20 minutes. After that the slides were washed three times in wash solution at 42°C for 5 minutes each.

Next 50µl of the following mix was added to each end of the slide:

5µl F2 (Detection reagent, Cambio)

500µl blocking reagent

1µl Texas Red (Vector)

The slides were covered with plastic coverslip and incubated at 37°C for 20 minutes. The slides were then washed three times in wash solution at 42°C for 5 minutes each. Then the slides were dehydrated using an ethanol series (70%, 80%, 95% and 100%v/v) and air dried. DAPI was prepared with 1µl DAPI, 9µl distilled water and 300µl mounting medium (Vectashield, Vector). 25µl of DAPI was added to each end of the slide. A glass coverslip was placed at each end ant the slides were visualised under Olympus BH2 microscope.

2.4 RESULTS

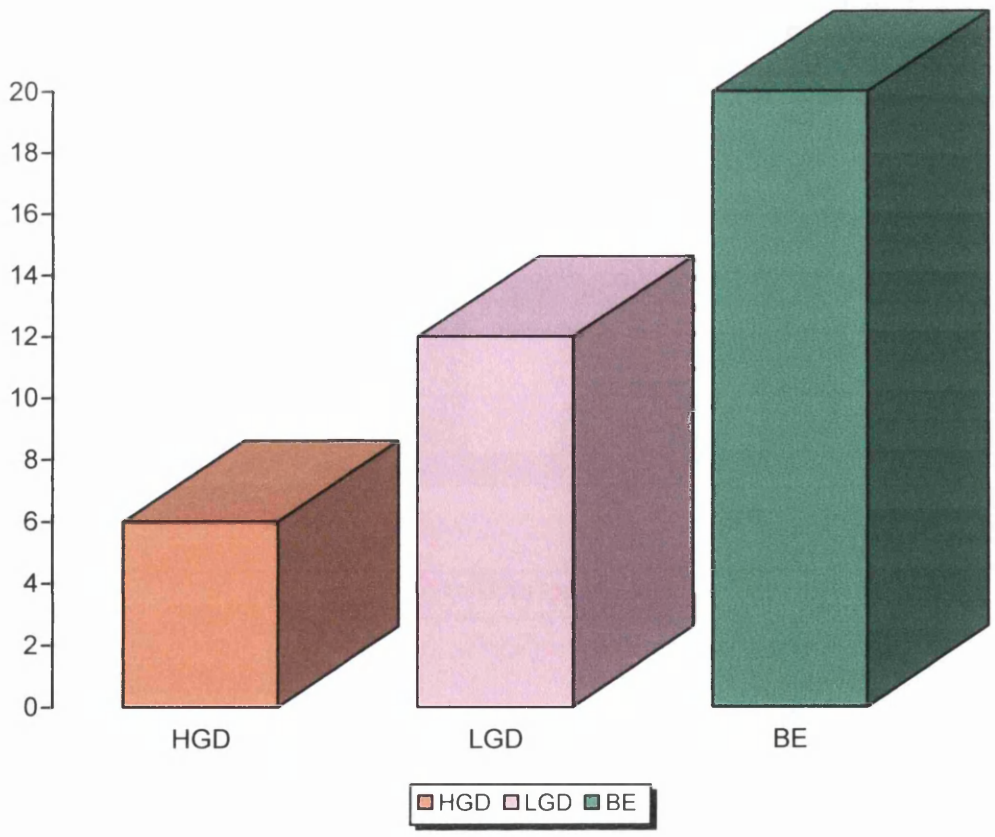
2.41 GENERAL DATA ON CELL CULTURE

Biopsies were obtained from 38 different patients with BE or BE with various grades of dysplasia. On an average 3-4 biopsies were obtained from each patient. Depending on the number and size of biopsies, cultures were initiated in 3-5 different flasks from each patient. Cultures were initiated exclusively by explant technique in 33 patients whilst both explant and enzymatic technique were used to intiate culture in the remaining 5 patients. Of the 38 patients 30 were male and 8 were female.12 of them had long segment Barrett's and 26 had short segment Barrett's. 6 patients had high grade dysplasia, 12 had low grade dysplasia and 20 had Barrett's metaplasia with no dysplasia (see fig 2.4). The mean age of the patients was 64 years (63.89).

FIGURE 2.4A SHOWING PATIENT PROFILE

	MALE	FEMALE	TOTAL
BE	15	5	20
LGD	9	3	12
HGD	6	0	6
LSB	9	3	12
SSB	21	5	26

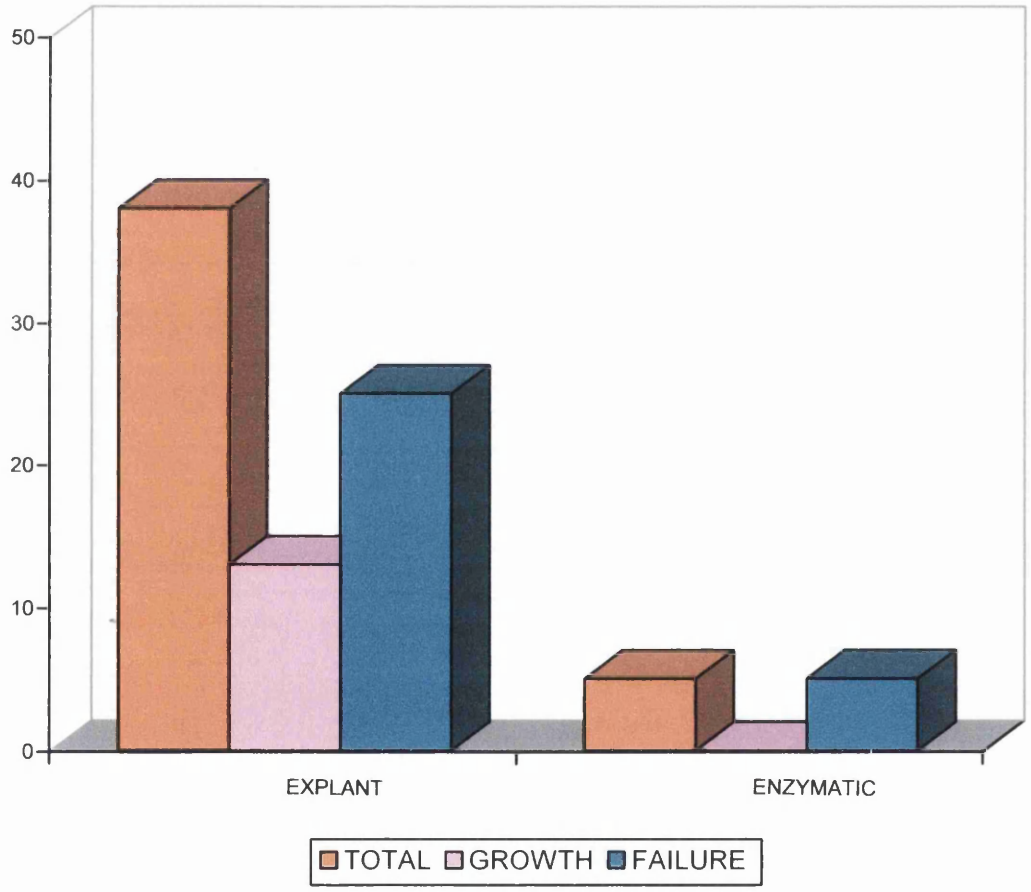
FIGURE 2.4B SHOWING THE DISTRIBUTION OF THE GRADES OF DYSPLASIA IN PATIENTS WITH BE.



2.42 EXPLANT TECHNIQUE

Cultures were initiated in 38 patients with the explant technique. Of these, samples from thirteen patients were successfully cultured while samples from twenty five patients either failed to grow or were lost to infection (see fig 2.5).

FIGURE 2.5 SHOWING THE OVERALL RESULT OF BE CELL CULTURE WITH EXPLANT AND ENZYMATIC TECHNIQUE



2.43 MORPHOLOGY AND GROWTH

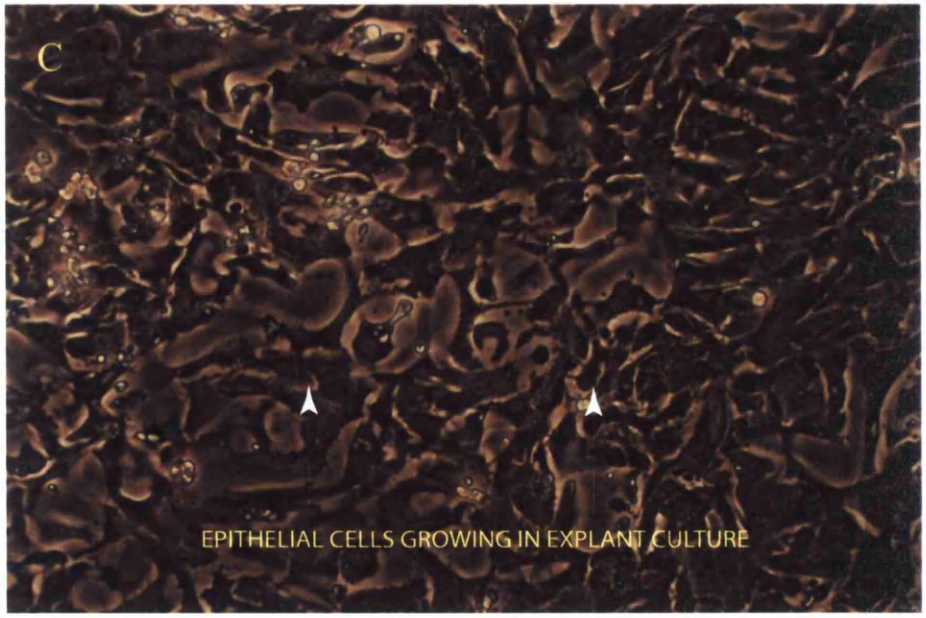
The general cell morphology and growth were assessed using routine microscopy. On microscopic examination multiple outgrowths of small epithelial cells were seen within the first three to four days. The outgrowths then spread out into a monolayer sheet of cells covering the surface of the flask. Morphologically the cells appeared polygonal in shape with irregular cytoplasmic processes (see figure 2.6). The epithelial nature of the cells was confirmed by the characteristic morphology of the cells. No special staining was performed to confirm the epithelial nature of the cells. The cultures were deemed positive when they could be maintained in monolayer for greater than 6-8 weeks or when they filled up more than 50% of the surface area of the flask. Though most of the cultures could be grown in monolayer, not all of them could be successfully sub-cultured (only two cultures could be sub-cultured once). They were either lost due to fibroblast overgrowth (recognised by their spindle shaped morphology) or were lost due to senescence. Although some of the cultures could be sub-cultured once, the cells progressively increased in diameter during second passage and failed to show attachment and growth. A total of 179 cultures were initiated from 38 patients. Of the 179 cultures 27 were successful whilst 98 were lost to infection and 54 failed to grow (see fig 2.7). Possible associations between the growth characteristics (ability to obtain positive) cultures and various culture conditions, gender, age and grade of dysplasia were analyzed using the chi-square test. Exact *P*-values were calculated using stata software (Stata Corporation USA).

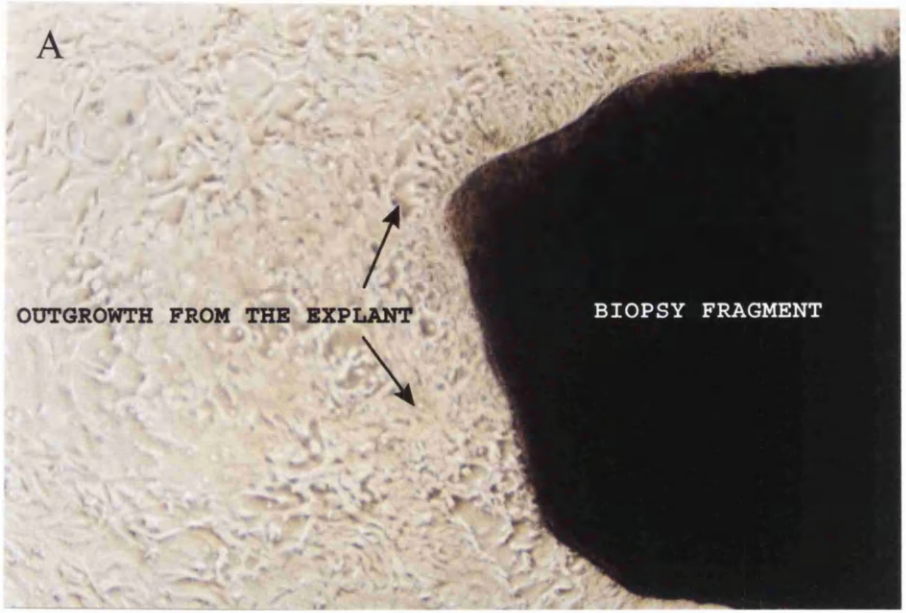
FIGURE 2.6 Showing Barrett's cells in culture.

A. low power picture showing biopsy fragment with cell outgrowth from the edge.

B. high power picture showing polygonal cells growing out from explant

C. Cultured Barrett's oesophageal cells at near confluence.





2.44 SEX AND GROWTH CHARACTERISTICS

35 samples from eight female patients and 144 samples from thirty male patients were used to initiate cultures (see fig 2.8). Of the 35 samples from the female patients two samples (from the same patient) were successfully cultured while the rest failed either due to infection or inability to grow. Similarly of the 144 samples from male patients only 25 showed growth, 81 were infected while 38 failed to grow. Successful cultures were more common in male patients compared to female patients. Though there was higher yield of positive cultures in male patients it was not found to be statistically significant (p value of = 0.084).

2.45 GROWTH CHARACTERISTICS AND HISTOLOGY

The maximum proportion of positive cultures was seen in patients with BE whilst patients with HGD had the lowest proportion of positive cultures. The rate of infection was the highest in patients with HGD and the least in patients with BE (see figure 2.9). The proportion of samples which failed to grow was highest in patients with LGD and the least in patients with HGD. Statistical analysis did not show any significant relationship between the grade of dysplasia and the ability of the cells to grow (p value =0.158 using chi-square test).

2.46 GROWTH CHARACTERISTICS AND AGE

The maximum proportion of growth was seen in the age group 31-40 years and the lowest proportion was seen in the age group 81-90 (0%). The highest proportion of infection was seen in the age group 81-90 while patients in the age group 51-60 years had the highest proportion of samples which failed to grow (see figure 2.10). The ability to obtain positive culture did not however show a definite pattern of

FIGURE 2.7 SHOWING THE NUMBER OF CULTURES INITIATED BY EXPLANT TECHNIQUE USING A TOTAL OF 179 SAMPLES

EXPLANT	NUMBER	PERCENT
GROWTH	27	15.08
INFECTION	98	54.75
NOGROWTH	54	30.17
Total	179	100.00.

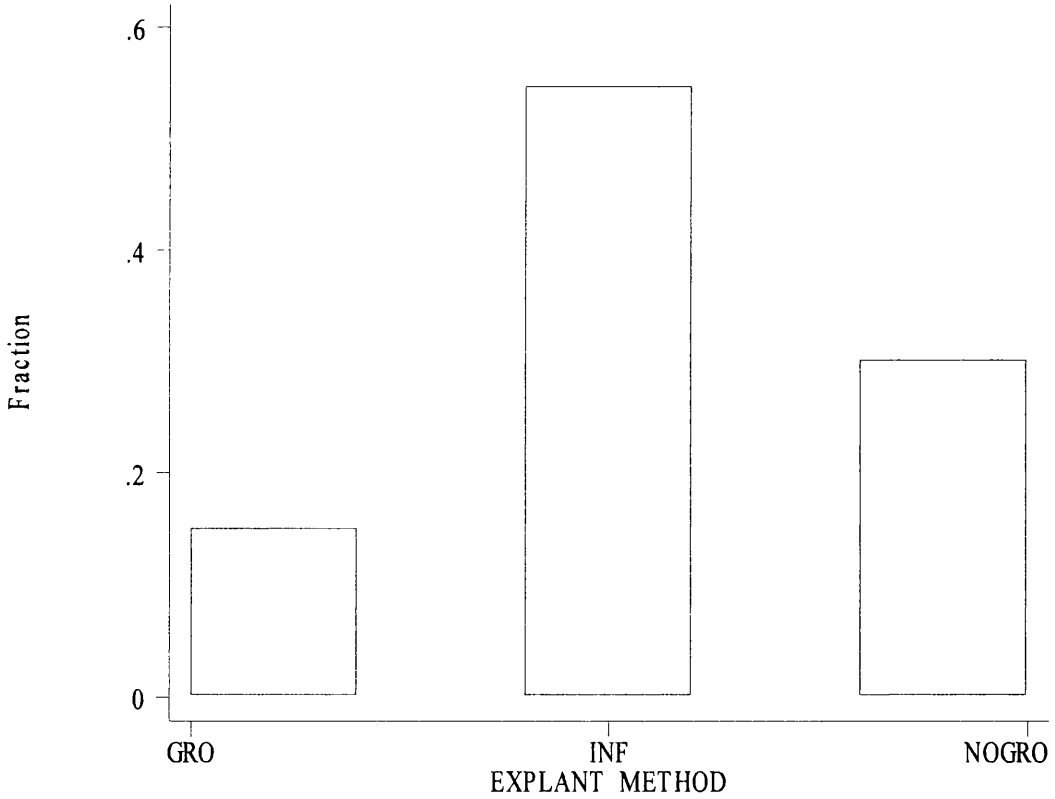


FIGURE SHOWING THE RESULTS OF THE EXPLANT TECHNIQUE

FIGURE 2.8 SHOWING THE RELATIONSHIP BETWEEN SEX AND GROWTH CHARECTERISTICS OF THE CULTURES

EXPLANT	FEMALE		MALE		TOTAL	
	NO.	%	NO.	%	NO.	%
GROWTH	2	5.7	25	17.4	27	15.1
INFECTION	17	48.6	81	56.3	98	54.8
NOGROWTH	16	45.7	38	26.4	54	30.2
TOTAL	35		144		179	

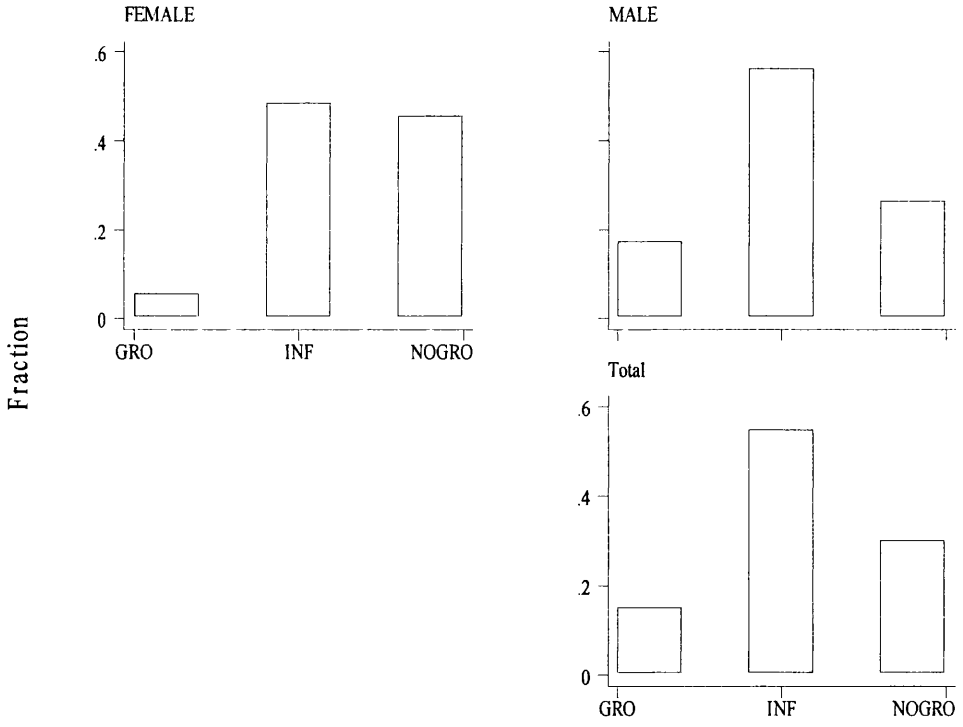


FIGURE 2.9 SHOWING THE RELATIONSHIP BETWEEN THE GRADES OF DYSPLASIA AND GROWTH PATTERN

EXPLANT	BE		HGD		LGD		TOTAL	
	NO.	%	NO.	%	NO.	%	NO.	%
GROWTH	19	19.8	2	7.7	6	10.5	27	15.1
INFECTION	46	47.9	21	80.8	31	54.4	98	54.8
NO GROWTH	31	32.3	3	11.5	20	35.1	54	30.2
TOTAL	96		26		57		179	

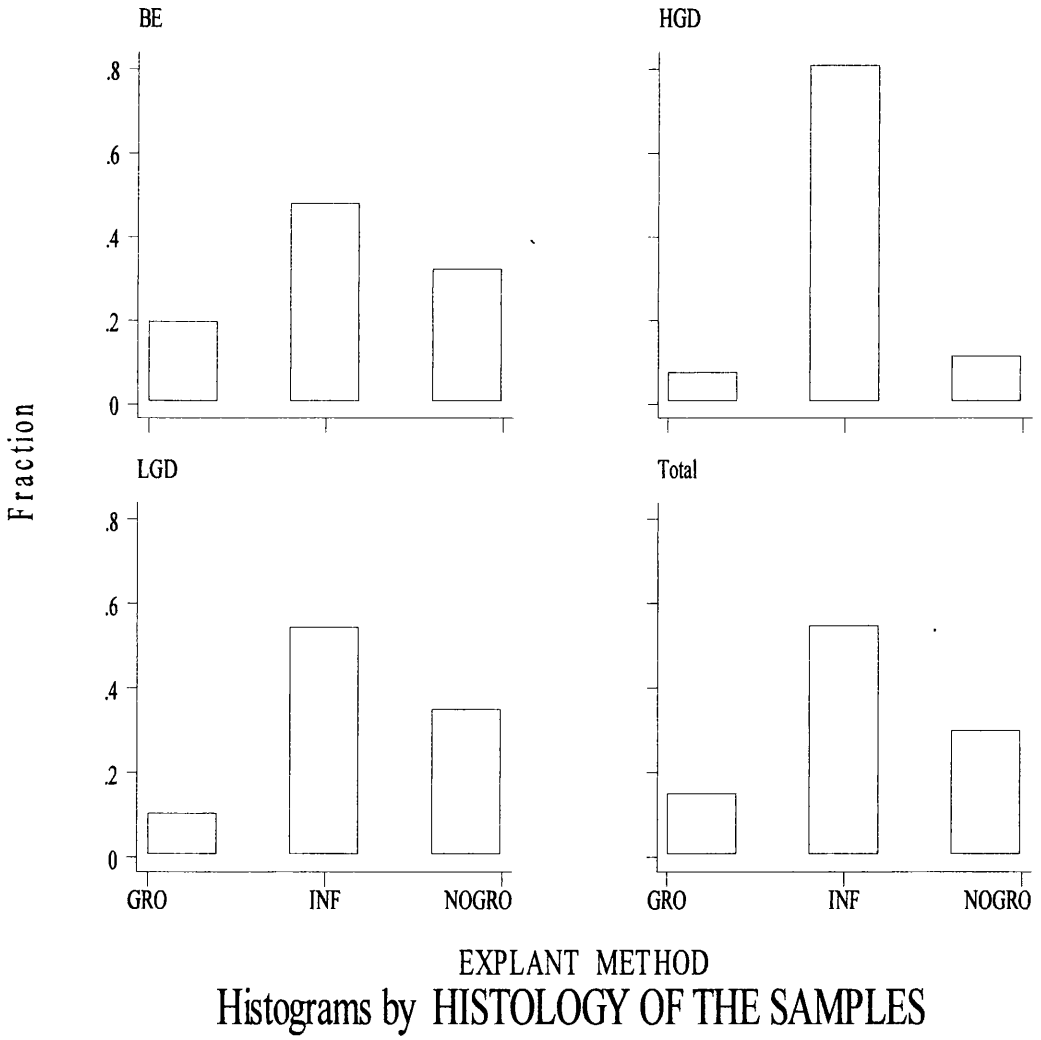
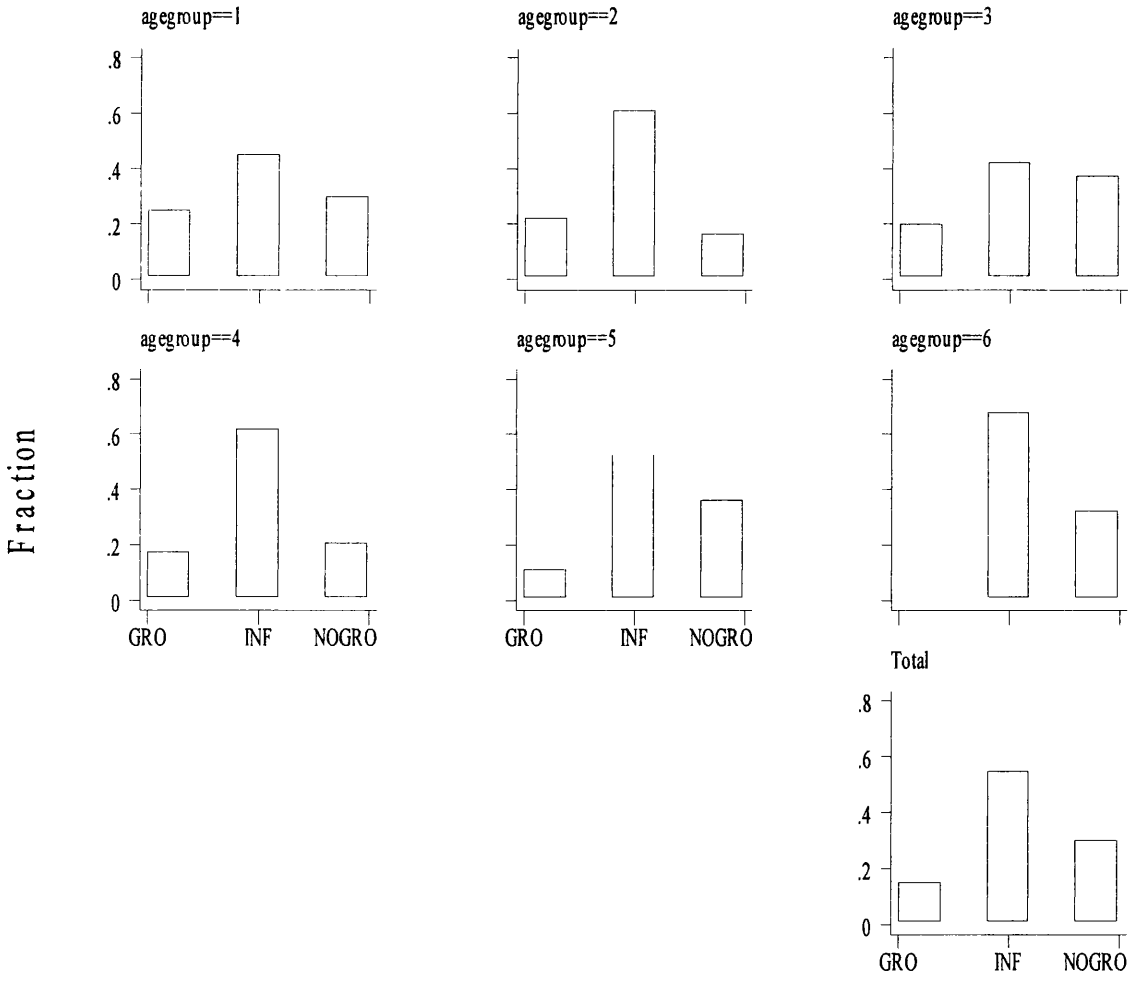


FIGURE 2.10 SHOWING THE RELATIONSHIP BETWEEN THE AGE GROUP AND GROWTH PATTERN

AGE GROUP	GROWTH		INF		NO GRO		TOTAL	
	NO.	%	NO.	%	NO.	%	NO.	%
1 (31-40)	5	18.5	9	9.2	6	11.1	20	11.2
2 (41-50)	4	14.8	11	11.2	3	5.6	18	10.1
3 (51-60)	8	39.6	17	17.4	15	27.8	40	22.4
4 (61-70)	6	22.2	21	21.4	7	12.9	34	18.9
5 (71-80)	4	14.8	19	19.4	13	24.1	36	20.1
6 (81-90)	0	0	21	21.4	10	18.5	31	17.3
TOTAL	27		98		54		179	



EXPLANT METHOD

Histograms by AGE GROUP OF THE PATIENT

relationship to the age group of the patient. Statistical analysis did not show any significant relation between the age of the patient and its ability to grow in vitro (p-value =0.104). Hence it is unlikely that the age group of patient's had a significant influence on the growth.

2.47 EFFECTS OF ANTIBIOTIC CONCENTRATION ON THE GROWTH PATTERN

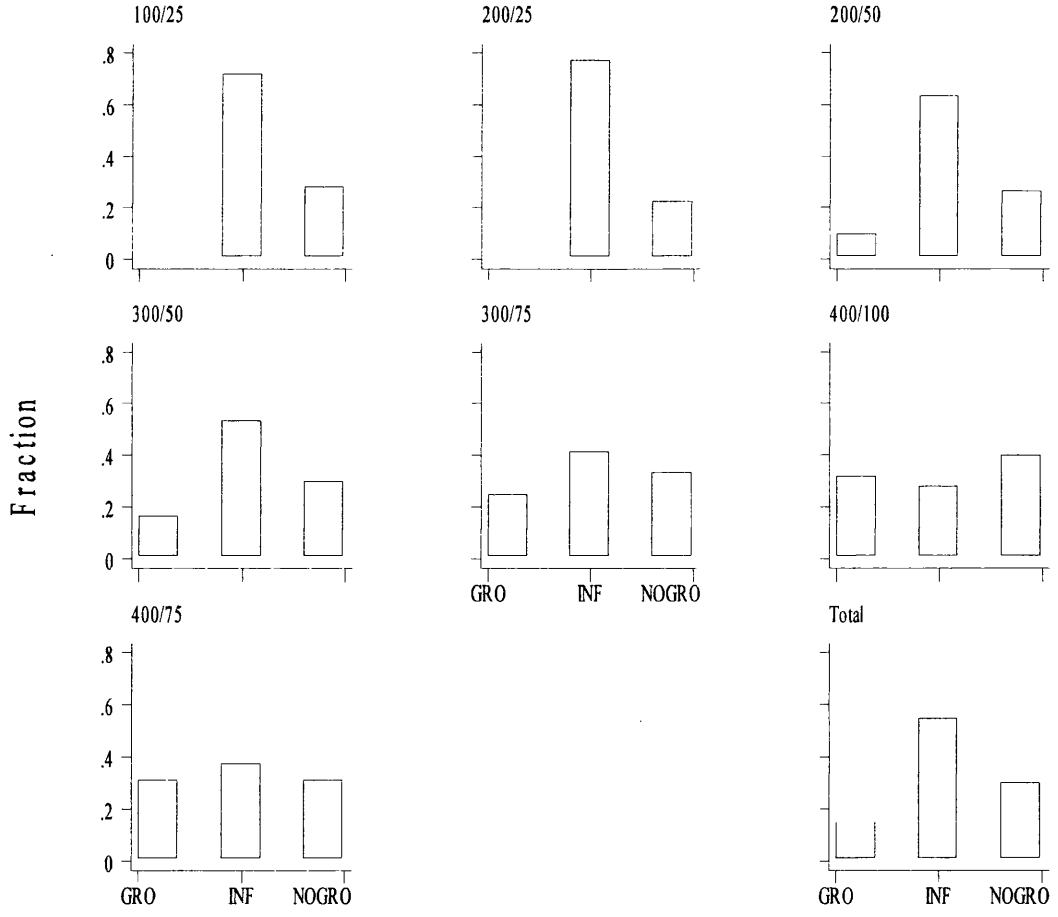
Different concentrations of various antibiotics (namely penicillin, streptomycin and amphotercin-B) were used to control infection. The various concentrations used are shown in the table (figure 2.11). It is obivious from the histogram, that with lower concentration of antibiotics the rate of infection was high. Increasing the concentration of the antibiotics decreased the rate of infection.

Maximum successful cultures were obtained with the antibiotic concentration of penicillin and streptomycin in the range of 300-400 Units and amphotercin in the range of 50-75μg. Statistical analysis (chi-square test using stata) showed that the concentration of the antibiotics had a significant effect ($p<0.005$) on the overall growth pattern (ie growth, inhibition or infection). Individual analysis of the effects of antibiotics on growth and infection rate using chi-square test revealed a stastically significant association (p values of 0.002 and 0.003 respectively). Though there was apparent increase in the inhibitory effect of increasing concentration of antibiotics on growth of the cells this was not found to be statistically significant ($p= 0.912$). The **enzymatic technique** was used to initiate cultures in samples obtained from five different patients. Most of the samples were either lost to infection or failed to grow. This technique was used only during the initial part of our study. Owing to the poor results it was not used subsequently.

FIGURE 2.11 SHOWING THE RELATIONSHIP BETWEEN THE ANTIBIOTIC CONCENTRATION AND GROWTH PATTERN

PEN-STREP	GROWTH		INFECTION		NO GROWTH		TOTAL	
AMP- U/ml & µg/ml	NO.	%	NO.	%	NO.	%	NO.	%
100 /25	0	0	23	23.5	9	16.7	32	17.9
200 /25	0	0	17	17.4	5	9.3	22	12.3
200/50	3	11.1	19	19.4	8	14.8	30	16.8
300/50	5	18.5	16	16.3	9	16.7	30	16.8
300/75	6	22.2	10	10.2	8	12.9	24	13.4
400/100	8	29.6	7	7.1	10	18.5	25	13.9
400/75	5	18.5	6	12.1	5	9.3	16	8.9
Total	27		98		54		179	

Pearson chi2(12) = 28.3006 P value = 0.005



EXPLANT METHOD

Histograms by ANTIBIOTIC CONCENTRATION

2.5 INTERPHASE FISH STUDIES

Interphase FISH was used to detect numerical chromosomal abnormalities from the cultured samples. Interphase FISH was used because it was extremely difficult to obtain proper metaphase spreads from the cultured cells. All the slides were coded before counting. The hybridization signals were counted in about 300-400 interphase nuclei per sample. Nuclei lacking hybridization signals were excluded from analysis in order to avoid erroneous results caused by inadequate hybridization. Nuclei showing over-lapping, damage or smearing, or those covered with bacteria were not included in the analysis. All the samples were visualized with an Olympus BX50 epi-fluorescence microscope with single and multiple band pass filters for simultaneous detection of DAPI (white), propidium iodide (orange), Cy-3 (red) and FITC (green). All the images were captured with a camera coupled to the Mac-probe version 4.1 software (Applied imaging software, Newcastle Upon Tyne, UK).

Numerical abnormalities were detected from samples obtained from five different patients. Of these three were BE with no dysplasia, one LGD and one HGD. Although samples from 13 patients were successfully cultured it was not possible to obtain cells for interphase study from all of the cultured samples. This was because at the beginning of the study many of the cultures were lost as they were either left in culture for a long period of time in order to obtain long-term culture or were lost in an attempt to obtain metaphase spreads. Normal controls were not used in the study as it was extremely difficult to obtain cultures from normal biopsy specimens. Each half of the biopsy specimen was divided into two halves with one half sent for histopathological analysis whilst the other half used for culture and interphase FISH analysis using centromeric probes for the chromosomes four and chromosome eight.

The numerical chromosomal abnormalities of chromosomes four and eight encountered in five different patients are shown in table 2.4 and table 2.5. Presence of

more than two signals was interpreted as gain whilst fewer than two signals were interpreted as loss of chromosome. All the specimens showed aneusomy ranging from 0.75 – 20%. Aneusomy was seen for both chromosomes four and eight. Most of the samples showed both gain and loss of chromosomes (see figure 2.12 and table 2.6) although hyperploidy was the predominant change for both chromosomes four and eight. Hyperploidy of chromosome four was seen in all cases ranging from 0.75% - 18.2%. Hyperploidy was seen in cases with both BE (12.75%) and HGD (12.89%). Loss of chromosome four was seen in a range of 0.5 – 1.84% of the counted cells (see figure 2.13). Similarly hyperploidy seemed to be the major change with chromosome eight. The total gain of chromosome eight varied from 0.29% - 13.2%. Loss of chromosome eight ranged from 0.25% - 0.86% (see figure 2.14). Hyperploidy of chromosome eight was predominant in one particular patient with HGD. Hyperploidy of both chromosome four and eight was common in our study. Owing to the small number of samples included in the study and lack of controls, it is extremely difficult to interpret the significance of these changes. The study does however suggest that hyperploidy of chromosome four could be an early change in the progression of BE to adenocarcinoma.

2.6 DISCUSSION

Primary culture of epithelial cells from human gut and oesophagus can be a difficult process. Establishment of long-term cell lines is hard, labour intensive, time-consuming, and unpredictable work with a very low success rate. Studies have shown that cells are more difficult to grow in vitro in the early stages of cancer than in the later stages.

TABLE 2.4 SHOWING THE RESULTS OF INTERPHASE FISH WITH CEP PROBE FOR CHROMOSOME FOUR

PT .NO	HISTOLOGY	NO OF CHROMOSOME COPIES				NO OF CELLS COUNTED
		1(%)	3(%)	4(%)	4+ (%)	
A	BE	5(1.25)	**51(12.75)**	04(1.00)	0	400
B	BE	3(0.75)	**38(09.50)**	02(0.50)	0	400
C	BE	2(0.57)	**27(07.71)**	03(0.86)	0	350
D	LGD	2(0.50)	**03(00.75)**	00	0	400
E	HGD	7(1.84)	**49(12.89)**	20(5.26)	2(0.53)	380

TABLE 2.5 SHOWINGTHE RESULTS OF INTERPHASE FISH WITH CEP PROBE FOR CHROMOSOME EIGHT

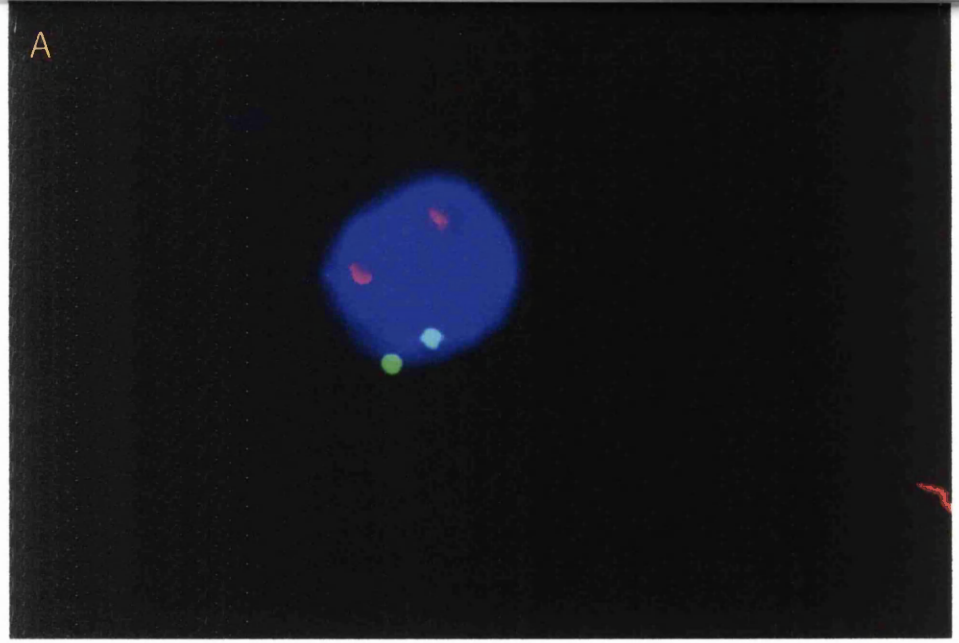
PT .NO	HISTOLOGY	NO OF CHROMOSOME COPIES				NO OF CELLS COUNTED
		1(%)	3(%)	4(%)	4+ (%)	
A	BE	3(0.75)	**4(1.00)**	01(0.25)	0	400
B	BE	2(0.50)	**03(0.75)**	02(0.50)	0	400
C	BE	3(0.86)	**01(0.29)**	0	0	350
D	LGD	1(0.25)	**02(0.50)**	0	0	400
E	HGD	3(0.79)	**41(10.80)**	9(2.40)	0	380

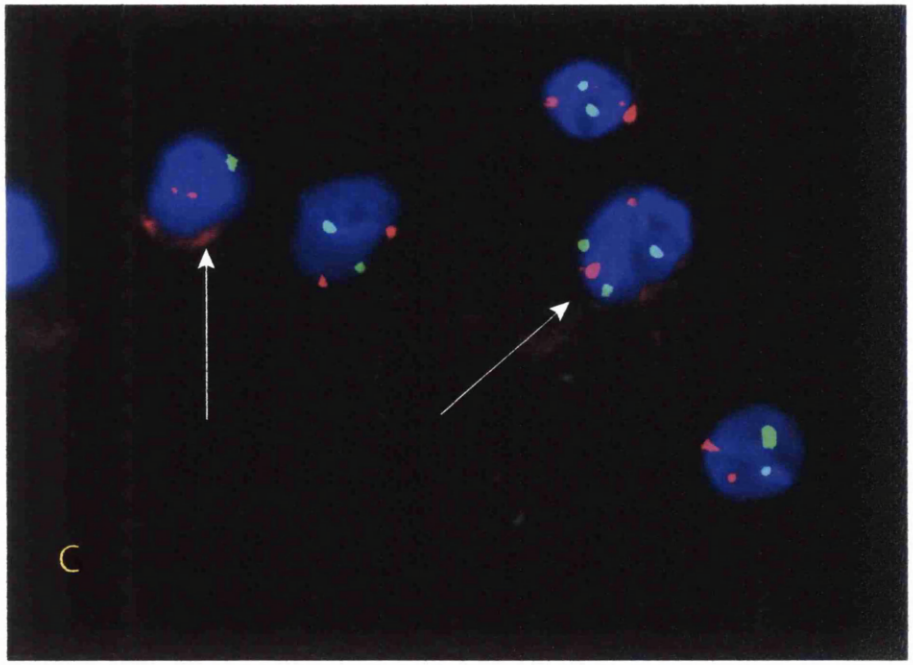
FIGURE 2.12 SHOWING RESULTS OF INTERPHASE FISH

A. Cell with two signals of chromosome four (red) and eight (green).

B. picture showing cell with four signals of chromosome four (red) and two signals of chromosme eight (green).

C. Picture showing cells with gain and loss of chromosome eight (green).





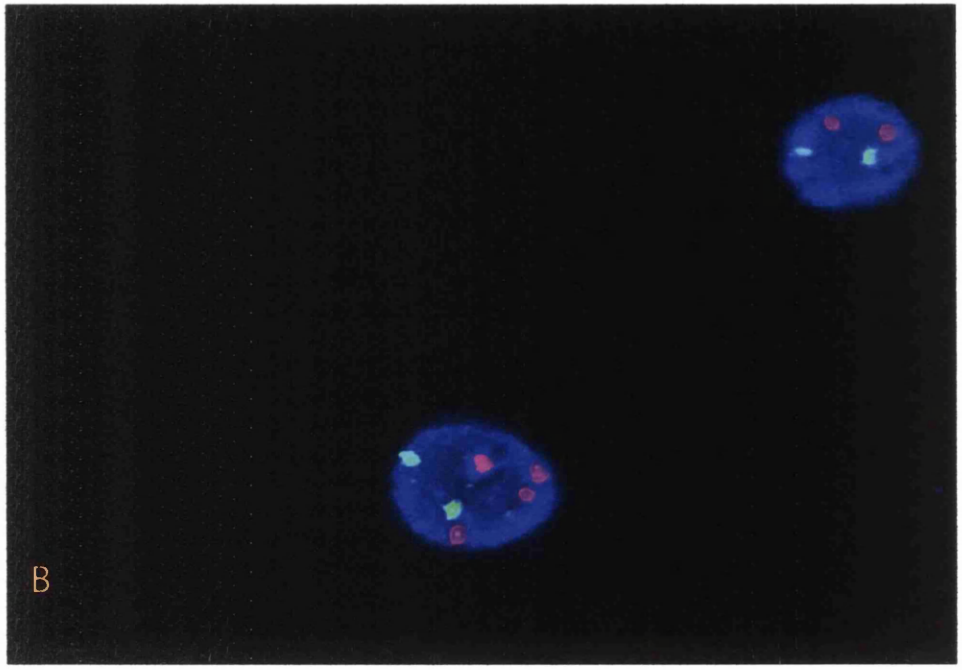


FIGURE 2.13 SHOWING CORRESSPONDING GAIN AND LOSS OF CHROMOSOME 4 IN FIVE DIFFERENT PATIENTS

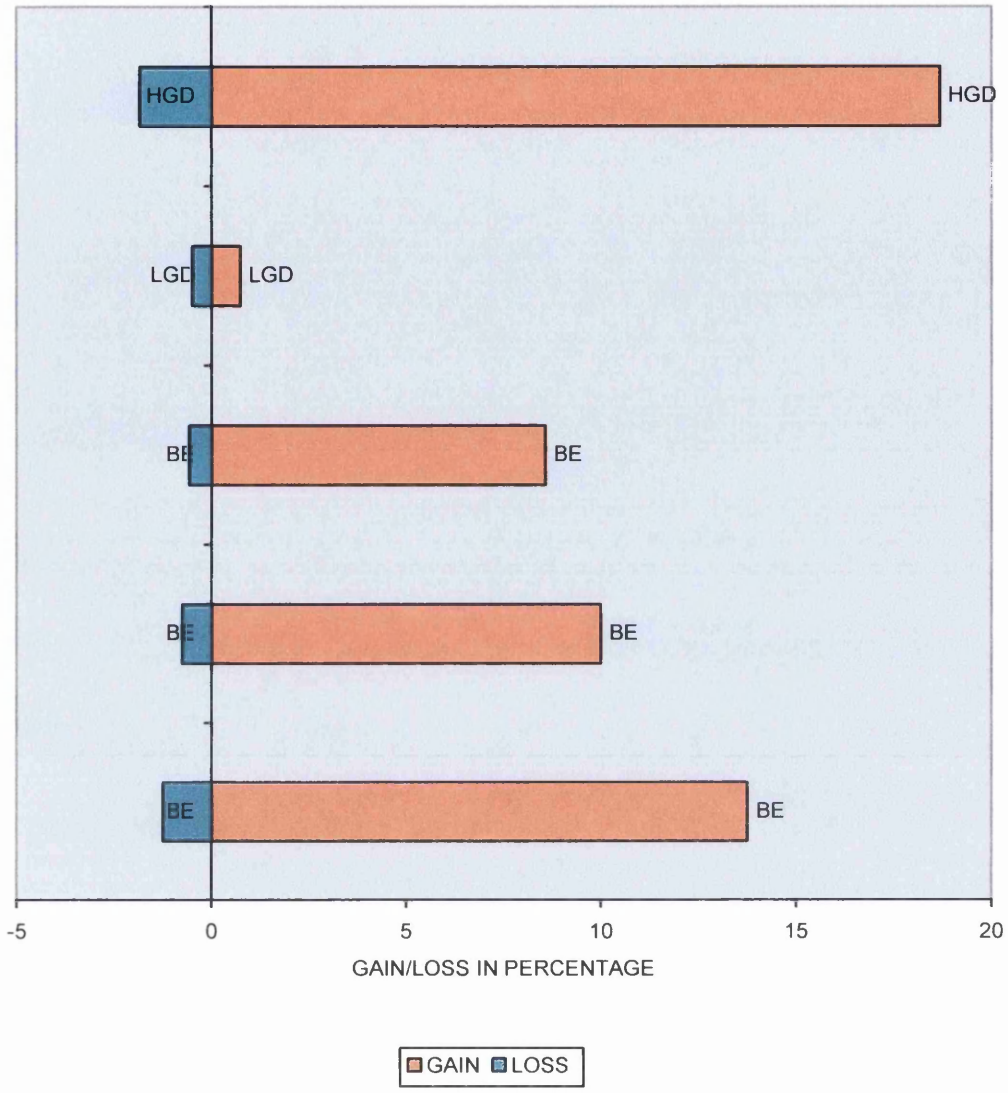


FIGURE 2.14 SHOWING CORRESSPONDING GAIN AND LOSS OF CHROMOSOME 8 IN FIVE DIFFERENT PATIENTS

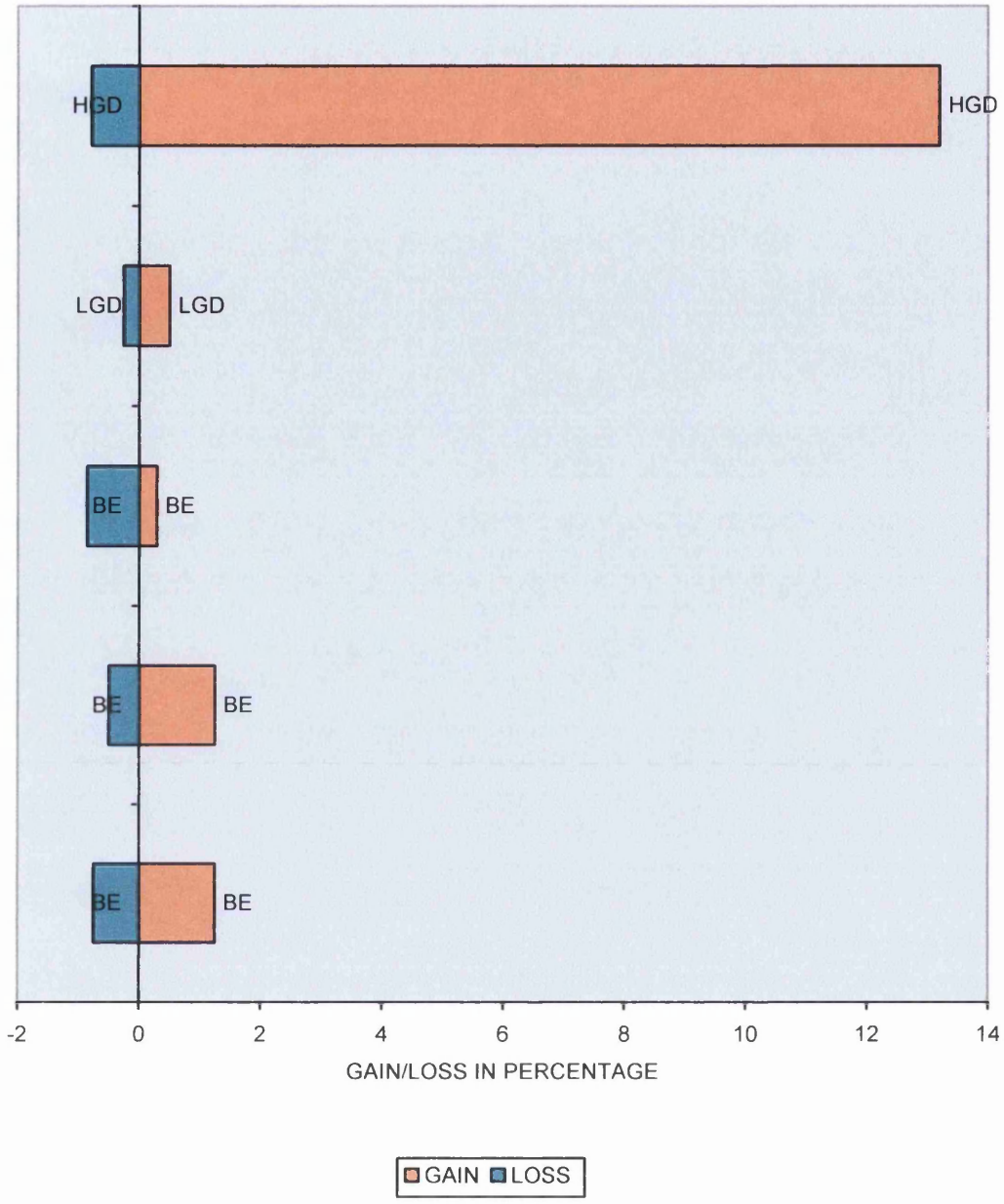


TABLE 2.6 SHOWING TOTAL GAIN AND LOSS OF CHROMOSOME FOUR AND EIGHT IN FIVE DIFFERENT PATIENTS

PT.NO	HISTOLOGY	CHROMOSOME 4		CHROMOSOME 8	
		% LOSS	% GAIN	% LOSS	% GAIN
A	BE	1.25	13.75	0.75	1.25
B	BE	.75	10	0.5	1.25
C	BE	0.57	8.57	0.86	.29
D	LGD	0.5	0.75	0.25	.50
E	HGD	1.84	18.68	0.79	13.20

Primary cell lines serve as an excellent tool to study the physiological and pathological processes occurring in the cells of their origin. Cultured cells provide the advantage of using human cells. The advantage of primary cell culture is that they are more representative of the organ under consideration. More importantly, primary cell lines from Barrett's oesophagus could serve as an excellent model to study the metaplasia-dysplasia–carcinoma sequence in vivo. Not only would it enable us to study the cytogenetic changes but also to evaluate the effects and role of various noxious substances (such as acid, pepsin, bile, trypsin) in the pathogenesis of BE and oesophageal adenocarcinoma. A review of the literature has shown that very few workers from the past have had success with primary culture of Barrett's oesophagus. Most of these studies describe methods of culture of BE.

Our results have been comparable to studies conducted in the past. Like most workers in the past we used the explant technique to culture our cells. We were able to obtain monolayer culture with an overall success of 34.2% which is comparable to most other studies in the past.

Previous studies have not noted any significant difference in cell growth based on histological grade of the specimen and sex of the patients. Similarly we did not record any significant difference in the ability of the cells to grow based on their histology. However there seemed to be a slightly better success rate in men than in women and in younger patients. The former could be because of the small number of women included in the study. We noticed a significant difference in the ability of the cells to grow based on antibiotic concentration (see fig 2.11). At lower concentrations (of antibiotics) there was a significantly high rate of infection. Increasing the concentration of antibiotics had a significant effect on decreasing the infection rates and improving the culture yield.

We had very limited success at obtaining serial passages. Attempts to sub-culture these cells resulted in their senescence except in two samples (which could be passaged twice). Despite using the same technique as described in previous studies we were unable to obtain serial passages. This clearly shows that the technique is unpredictable and not easily reproducible. Most of our positive cultures lasted for 6-8 weeks. In the early part of the study most of the cultures were lost to senescence as they were left for a longer period of time or disaggregated to obtain subcultures.

Towards the end of the study the cell cultures were trypsinised at an early stage in order to obtain cells to perform interphase FISH study. It was not possible to obtain or process more samples due to constraints of time and lack of adequate quantity of biopsy sample.

The short term primary culture technique could be used for several other studies such as interphase FISH. However they would not be suitable for short term toxicity studies as they are not easy to obtain in a reproducible manner, which would make it difficult to standardize testing conditions.

Senescence of the cells was one of the major factors affecting the long term survival of the Barrett's cells in vitro. This is probably because the cells undergo terminal differentiation. Explantation of the cells itself induces trauma which can shorten the life of the cells. The cells explanted from organisms and grown in vitro have a relatively brief replicative lifespan compared to the impressively long and robust replication they have in vivo. This is possibly due to the fact that tissue organization, hormone levels and other homeostatic mechanisms play an important role in growth, which are absent in vitro (Rosen 1978; Rubin 1997; Elsasser *et al.,* 1998).

Cell anchorage has been thought to be one of the important factors in survival of the cells. Studies have shown that cell anchorage not only provides structural anchorage but also mediates pivotal survival signals for the survival of the cell. Survival of most normal epithelial cells requires adhesion to the basement membrane. Loss of cell-basement membrane contacts results in death of such cells by apoptosis, a phenomenon known as anoikis (Meredith *et al.,* 1993; Frisch *et al.,* 1994). Anoikis is likely to be one the mechanisms terminating the life of cells in vitro.

One other factor responsible for the limited replicative life span of the cell in culture is the absence of the enzyme telomerase which maintains the length of telomeres. The absence of this enzyme can lead to critical shortening of the protective ends of chromosomes. Studies have shown that cell multiplication is dependent on a minimal telomere length. Activation of the telomerase enzyme is one of the methods to immortalize cell lies. More recently immortalised Barrett's cell lines have been generated by activation of the telomerase enzyme(Palanca-Wessels *et al.,* 2003).

An increasing number of changes involving oncogenes, tumor suppressor genes growth factors, cell cycle related proteins and chromosomal ploidy have been described in patients with oesophageal adenocarcinoma and Barrett's oesophagus.

To date only a limited number of studies have investigated the cytogenetic changes in Barrett's oesophagus. A number of workers have detected loss and gain of several chromosomes using various methods such as flow cytometry, CGH and interphase FISH techniques. Increased frequencies of ploidy with progression to dysplasia and adenocarcinoma have been described in the past. The results of most of these studies have been inconsistent and none of the markers described so far can be reliably used to predict the progression of BE to oesophageal adenocarcinoma.

Studies performed by our group using CGH showed that there was amplification of chromosome four and chromosome eight in patients with BE (Croft *et al.,* 2002). These changes were seen mostly in patients with high grade dysplasia. CGH detects changes when present in high proportion of the cells. Changes present in a low proportion of cells can easily overlooked by CGH. We therefore used interphase FISH technique to see if these changes occurred in the early stages of BE.

Interphase FISH is more sensitive and can detect changes when present in smaller proportion of the cells. The initial goal of our study was to obtain metaphase spreads from growing cells which would enable us to perform a complete karyotyping (using whole chromosome FISH). As it was impossible to obtain metaphase spreads from the growing cells, interphase FISH technique was used to analyze the chromosomal aberrations.

Our studies did confirm the changes (amplification of chromosome four and chromosome eight) previously detected by our CGH studies. These changes (hyperploidy of chromosome four) were seen in patients with HGD as well as patients with BE. This suggests that aneuploidy could be an early event in tumorigenesis. Hyperploidy of chromosome four both in early and late stages of Barrett's suggests that it could be involved in the neoplastic progression of BE. Studies in the past have recorded both amplification and loss of chromosome four. It is difficult to make a

sensible interpretation of the results as the number of samples analyzed in the study was too small. A study with larger number and appropriate controls should be more conclusive (Doak *et al.,* 2003).

Our study shows that interphase FISH can be reliably used for detection of numerical chromosomal aberration in BE. Large numbers of samples could not be obtained in this study because of the constraints imposed by cell culture. Although cells can be obtained for interphase study by short term cell culture, it is difficult to use because of lack of reliability, reproducibility and simplicity. The only published study so far in which cell culture was used as method to investigate cytogenetic abnormalities was by Garewal *et al* (Garewal *et al.,* 1992). Although Wessels *et al* manged to generate long term cultures of BE their cytogenetic studies were based mostly on CGH and flow cytometry (Palanca-Wessels *et al.,* 1998). Studies by other worker have only described techniques to culture BE and no attempts have been made to perform cytogenetic analysis at the time of publication or subsequently. Clearly this shows that although BE cells can be cultured in short term and possibly long term it is highly unreliable and not easily reproducible. Lack of simplicity makes this technique unacceptable. Other techniques such as brush cytology and touch prep technique are much simple and less time consuming for obtaining cells for FISH analysis. Unlike cell culture they can be relied upon and analyzed quickly. In view of the difficulty encountered in obtaining a continuous cell line from BE it was not possible achieve some of the goals that were originally planned. We decided to use an alternative model for our further studies which would be a continuous cell line that would be immortal and would replicate with ease and at the same time bear some similarities to the tissue of origin. The OE33 cells, which are derived from Barrett's oesophageal adenocarcinoma were selected as they fulfilled our requirements. The next chapter briefly describes the OE33 cells and their characterization.

CHAPTER 3

CHARACTERISATION OF THE OE33 CELL LINES

3.1 INTRODUCTION

Cytogenetic analysis of tumor cells provides essential data regarding the origin, biology, behavior and prognosis of the tumor. Not only does it identify the lineage to which the cell belongs but also enables monitoring of cell lines for instability and variation, and to detect cross contamination (Freshney 1987). The information regarding solid tumours and carcinomas is limited owing to the technical difficulties in culturing the neoplastic cells and partly due to the karyotypic complexity of some of these cells. This chapter deals with the cytogenetic analysis of the OE33 cells which were generated by Rockett *et al* in 1997 from oesophageal adenocarcinoma cells(Rockett *et al.,* 1997).

3.2 CHARACTERISATION OF THE CELL LINES

3.21 MORPHOLOGY

This is the simplest and most direct technique to identify cells, although it is not the most reliable method. It is usually possible to identify epithelial from fibroblastic cells by their appearance. Epithelial cells are regular and polygonal with a clearly defined edge. The fibroblasts usually appear as bipolar or multipolar cells with the length usually being twice that of the width. Some times it may be difficult to identify the epithelial nature of the cells based on the morphology alone. There are certain structurally related molecules called cytokeratins which are ubiquitously expressed by both normal and experimentally altered epithelial cells. Identification of these cytokeratins with immunohistochemical techniques serves as a reliable marker for epithelial cells (Moll *et al.,* 1982).

3.22 CYTOGENETIC ANALYSIS

3.221 CHROMOSOME BANDING

Chromosomal alterations play an important role in neoplasia. A variety of genes like the oncogenes, the tumour suppressor genes and genes associated with apoptosis can all be affected by chromosomal deletions, translocations or amplifications. Identification of recurrent cytogenetic alterations can be of diagnostic and prognostic significance. The earliest karyotyping was performed by Hungerford et al in 1959 from peripheral blood cultures by using relatively crude techniques (Hungerford *et al.,* 1959). The chromosomal banding itself was first introduced by Caspersson *et al* in 1968 (Caspersson *et al.,* 1968). Over the years, cytogenetic techniques have made immense progress from simple banding techniques to advanced multicolour FISH (M-FISH) and spectral karyotyping (SKY) which are more sensitive and accurate.

Chromosome banding techniques have facilitated the precise identification of individual chromosomes. G-banding obtained by digesting the chromosomes with proteolytic trypsin followed by Giemsa staining still remains the the most widely used technique for routine chromosome analysis. The Giemsa staining results in a characteristic banding pattern which is unique for each chromosome (figure 3.1). Based on the size and position of the centromere, and the G-banding pattern, the individual chromosomes are identified. The distinctiveness of these banding patterns not only enables identification of specific parts of a chromosome but also to locate the sites of chromosomal breaks and translocations. The limitation of G-banding is that it cannot detect chromosome structural rearrangement involving segments smaller than a band (around 10 Mb) or complex structural rearrangements (Heim S 1995). Similarly staining with Quinacrine, a fluorescent dye that inserts (intercalates) between base pairs in the DNA helix, produces *Q bands.* Q banding is generally not preferred because Q bands tend to fade away with time.

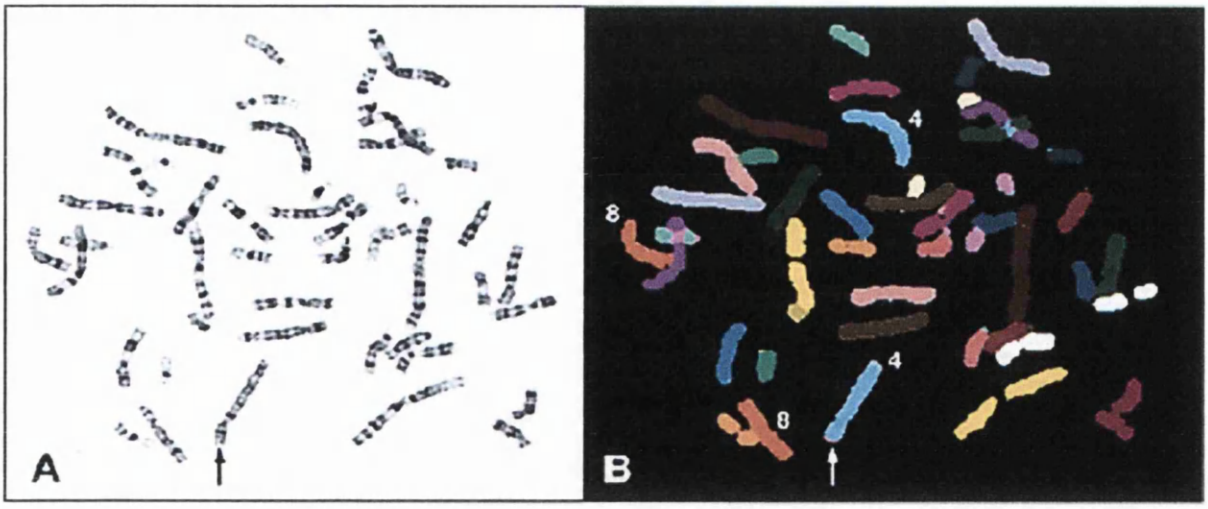


Figure 3.1 A. G banded metaphase B. The same Metaphase after SKY analysis [Adapted from Reid *et al., 1998*].

3.222 FISH

FISH (discussed in detail in chapter 2 section 2.65) has emerged as an important tool for the study of chromosome structure and genome mapping. FISH or chromosome painting allows the visualization of individual chromosomes in metaphase or interphase cells and the identification of both numerical and structural chromosomal abnormalities. Newer techniques like M-FISH (Multicolour FISH) and SKY (Spectral Karyotyping) enable painting and visualisation of all the 24 chromosomes in one experiment. Both M-FISH and SKY involve simultaneous hybridization of multiple chromosome painting probes, each tagged with a specific fluorochrome or fluorochrome combination resulting in the differential colour display of all the chromosomes, i.e. colour karyotyping (see figure 3.1). The advantage of this technique over the conventional G-banding include improved accuracy in the detection and characterization of translocations and an appreciable shortening of the time required for performing chromosome analysis. The major disadvantage is its poor sensitivity to detect aberrations like intra-chromosomal deletions, duplications and inversions (Le Beau 1996). The battery of G-banding, FISH methods and CGH used together can identify numerical alterations on a global basis, localize breakpoints

of translocations and deletions and help in-complete derivation of complex re-arrangements.

3.3 MATERIAL AND METHODS

3.31 OE33 CELL LINES

The OE33 cells were purchased from the EACC. The OE33 are oesophageal adenocarcinoma cells derived from a 73 year old female patient with poorly differentiated Barrett's adenocarcinoma. These cells which have been characterized by Rockett *et al.,* 1997 express epithelial cytokeratins and are tumourigenic in nude mice (Rockett *et al.,* 1997).

3.311 CULTURE OF OE33 CELLS

The OE33 cells (described in detail in chapter 3) were cultured in monolayer in 25 cm^2 tissue culture flasks (Nunclon) using:

- RPMI 1640 without glutamine (in vitro)
- 10%(v/v) Foetal calf serum (Gibco BRL)
- Penicillin 100IU/ml (Gibco)
- Streptomycin 100µg/ml (Gibco)
- Glutamine1mm/L (Gibco)

The cells were cultured in a humidified atmosphere of 5% CO_2, 95% air at 37°C. The cells were sub-cultured once they became confluent every four to six days. Fresh culture medium was added to the flask every two to three days. To split the cells the culture medium was removed by suction and discarded. About 4-5ml of 2.5% trypsin EDTA (Gibco BRL) was added. The fluid was washed around gently and discarded. The flask was rinsed again with 4-5ml of trypsin. The trypsin was discarded after 30-60 seconds so as to leave enough trypsin to just cover the bottom of the flask. The

flask was then transferred into the incubator at 37°C and left for about 5- 10 minutes. The monolayer was examined at frequent intervals under the microscope to see for signs of detachment from the surface of the flask, which was indicated by the rounding up of the cells. The flask was then gently tapped to aid the detachment of the cells. When the cell monolayer was completely detached and evenly dispersed about 5-10 ml of fresh medium was added. The cells were then split in the ratio of 1:3 or 1:4 depending on the density of the cells. Fresh culture medium was added to each flask to make up the quantity to 10 mls. The flasks were gassed with CO_2 and then incubated at 37°C in humidified atmosphere and 5% CO_2.

3.312 STORAGE OF OE33 CELLS

Cell stocks were preserved in liquid nitrogen to prevent contamination, and also to serve as backup in the event of technical problems such as bacterial infection or incubator failure. Cultures that had reached near confluence were trypsinised as already described. The cells were then re-suspended in the culture medium and centrifuged at 1500 RPM for 8 minutes. The supernatant was discarded and the pellet was re-suspended at approximately $5x10^6$ - $2x10^7$ cells/ml in 90% heat inactivated foetal calf serum (HIFCS) and 10% DMSO. DMSO is a cryoprotective agent, which prevents physical damage to the cell by ice crystals. The cells (1.5 ml of the suspension) were then transferred into sterile cryovials. The vials were labelled dated and placed in a bio-freezing (Bicell, Nihon freezer co Ltd) at -20°C overnight and then transferred to a cryomed liquid nitrogen freezer the next day.

3.313 THAWING OF CELL STOCK

To thaw a frozen cell stock the ampoule was placed in a 37°C water bath. The cell suspension was then transferred to a centrifuge tube containing pre-warmed culture medium and centrifuged at 1500RPM for 8 minutes. The supernatant was removed

and the cells were re-suspended in fresh culture medium. The cell suspension was then transferred to a $25cm^2$ flask and more culture medium was added to make up the total quantity to 10 mls. The flask was then gassed with CO_2 and incubated at 37°C in humidified atmosphere and 5% CO_2.

3.32 GIEMSA BANDING (G-BANDING)

The metaphase spreads were prepared from the OE33 cells as describer in Chapter 2 section 2.361. The protocol used for G banding was similar to the one described by Sumner *et al* (1971). G banding was performed as follows.

Aged slides (10-14 days) were put in 2 X SSC in a plastic Coplin jar at 60-65°C for 90 minutes (2 X SSC = sodium chloride 17.53g (0.3m) tri-sodium citrate 8.82g (0.03M) dissolved in 1 litre of deionised H_2O and made up to 100mls). The slides were removed from the bath and left to cool at room temperature. Thirty minutes before cooling 0.4% of trypsin (0.2g dissolved in 50mls saline) solution was made up. Once the slides cooled they were taken out of the 2 X SSC and put into normal saline (0.9 percent solution of sodium chloride) for 2 minutes. The slides were then drained off one by one by touching onto a filter paper, and then placed in a horizontal plane and flooded with the 0.4% trypsin for 1 minute. To stop the effect of trypsin, slides were put back into the normal saline to dilute it out (5-10 seconds). Slides were then transferred from the normal saline into phosphate buffer pH 6.8. They were then rinsed in the buffer once (in Coplin jar) and stained whilst the slides were still wet with 1 part Giemsa and 9 parts of the Gurr phosphate buffer (pH 6.8 0.005M phosphate) for 8 minutes. Finally they were washed with phosphate buffer (pH 6.8) and left to dry. The cells were counted under Olympus BH2 microscope with a triple band pass filter. About 50 metaphase cells were counted. Karyotyping was attempted with the Mac karyotype software.

3.33 FISH ANALYSIS USING WHOLE CHROMOSOME PROBES

Whole chromosome painting was done using Cambio FISH probes. The protocol used was the one supplied by the manufacturers (Cambio). The details of which are described in Chapter 2 section 2.362. Whole chromosome probes for all the chromosomes except 9, 21, 22 and Y chromosome were used. (due to lack of probes).

3.34 MICROTUBULIN AND CENTROSOME STAINING

The OE33 cells were seeded on a glass slide in a concentration of $1x10^5$/mm and were grown for over 24 hrs. The slides were washed by rinsing in ice cold PBS .They were then fixed with 90% methanol at -20°C for 30 minutes and subsequently in acetone at -20°C for 20 seconds. They were then rinsed in PBS/Tween (250 µl in 500 µl PBS). The slides were then incubated with primary antibody specific for γtubulin and ά tubulin as follows.

1. 50 µl of mono-clonal Anti - γ-Tubulin(T6557 diluted 1 in 200) was added to each slide and covered with plastic cover slips and incubated in a humidified chamber at 37°C for 2 hours. It was then rinsed in PBS/Tween at 37°C
2. Next 50 µl of anti mouse IgG (Fab Specific) TRITC conjugate (T7782 diluted 1 in 32) was added to the slides and covered with plastic cover slips and incubated in a humidified chamber at 37°C for 2 hours. It was then rinsed in PBS/Tween at 37°C.
3. 50 µl of monoclonal Anti - α - Tubulin Conjugate (F2168 diluted 1 in 100) was added to the slides and covered with plastic cover slips and incubated in a humidified chamber at 37°C for 1 hour. It was then rinsed with PBS/Tween.

4. The slides were then counterstained with DAPI (1µl DAPI+9µlH_2O+300µl vectorshield) and covered with glass cover slips and visualised under the microscope.

At least 200 cells were counted for each slide and were examined for abnormalities centrosome structure, mitotic spindles and nuclear morphology. The cenetrosomes were considered abnormal if the cells contained more than two copies per cell or if they were many times larger than the normal centrosome or if they were arranged in large patchy aggregates.

3.4 RESULTS AND DISCUSSION

3.41 METAPHASE SPREAD

Analysis for the chromosome number revealed gross polyploidy of the OE33 cell lines. Most of the cells had near tetraploid or more than tetraploid number of chromosomes. 100 metaphase cells were counted. The mean chromosome number for the OE33 cells was 90.27 with a modal karyotype number of 96. Repeat metaphase chromosomal counts after a year of initiating the cultures did not reveal any significant change in number.

3.42 G BANDING

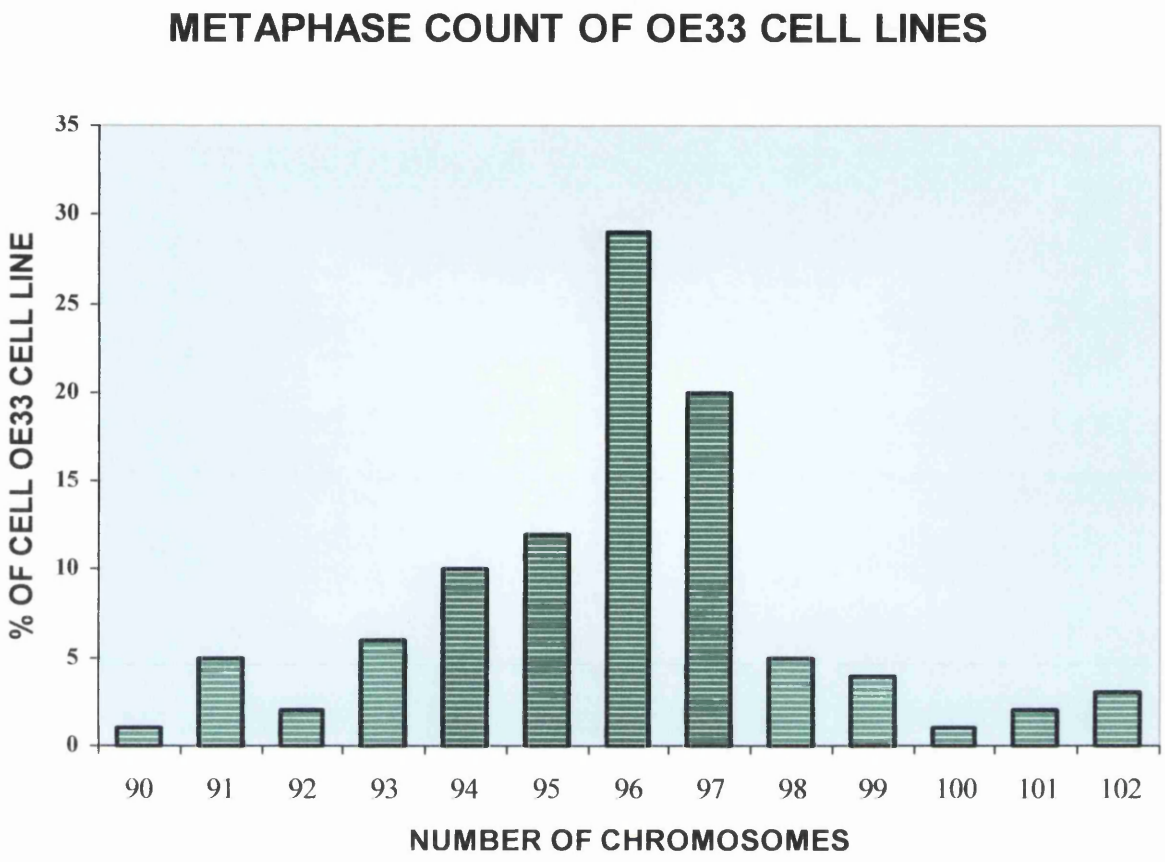
G banding using the trypsin technique is widely used in cytogenetic studies to identify chromosomes in metaphases. Giemsa banding produces a series of light and dark bands along the lengths of chromosomes, permitting the identification of chromosomes and chromosome regions. G banding of the OE33 cells revealed that there were multiple copies of most of the chromosomes (see figure 3.2). Many of the chromosomes also had gross structural abnormalities (probably deletions and translocations). Owing to gross polyploidy and complex structural abnormalities it

was virtually impossible to perform a complete Karyotype on these cells. Plate 3.1 shows G-banding of OE33 cells with attempted karyotyping in plate 3.2.

3.43 WHOLE CHROMOSOME PAINTING

To obtain more detailed information on the structural and numerical chromosomal abnormalities, FISH was performed using whole chromosome probes. Whole chromosome probes for chromosome 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 and X (except 9, 21, 22 and Y) were used in different combination. Each slide contained a cell suspension with metaphases at both ends. At each end two chromosome probes with different colour were used. In total four different chromosome probes were used for each slide. 20 metaphases were scored at each end of the slide and therefore 40 per slide per cell line. Metaphases were scored for the number of each chromosome and for the presence of translocation/ deletion. The results of the whole chromosome painting of the OE33 cells have been summarized in the table 3.1. Most cells showed multiple copies of the chromosome analyzed with complex translocations and possibly deletions.

FIGURE 3.2



NO OF CHROMOSOMES	90	91	92	93	94	95	96	97	98	99	100	101	102
NO OF CELLS	1	5	2	6	10	12	29	20	5	4	1	2	3

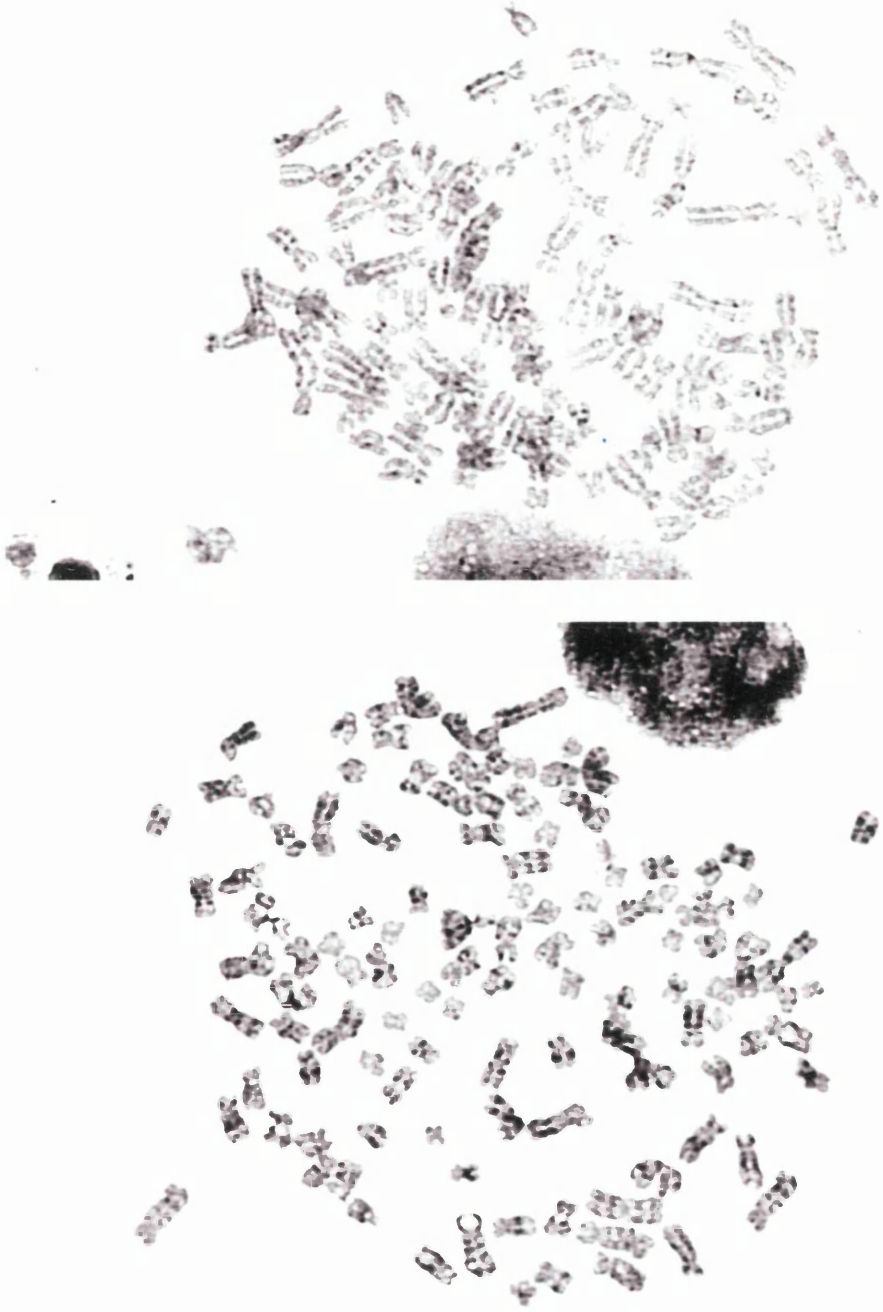


PLATE 3.1 Metaphase spreads from two different OE33 cells banded with the conventional GTG banding technique.

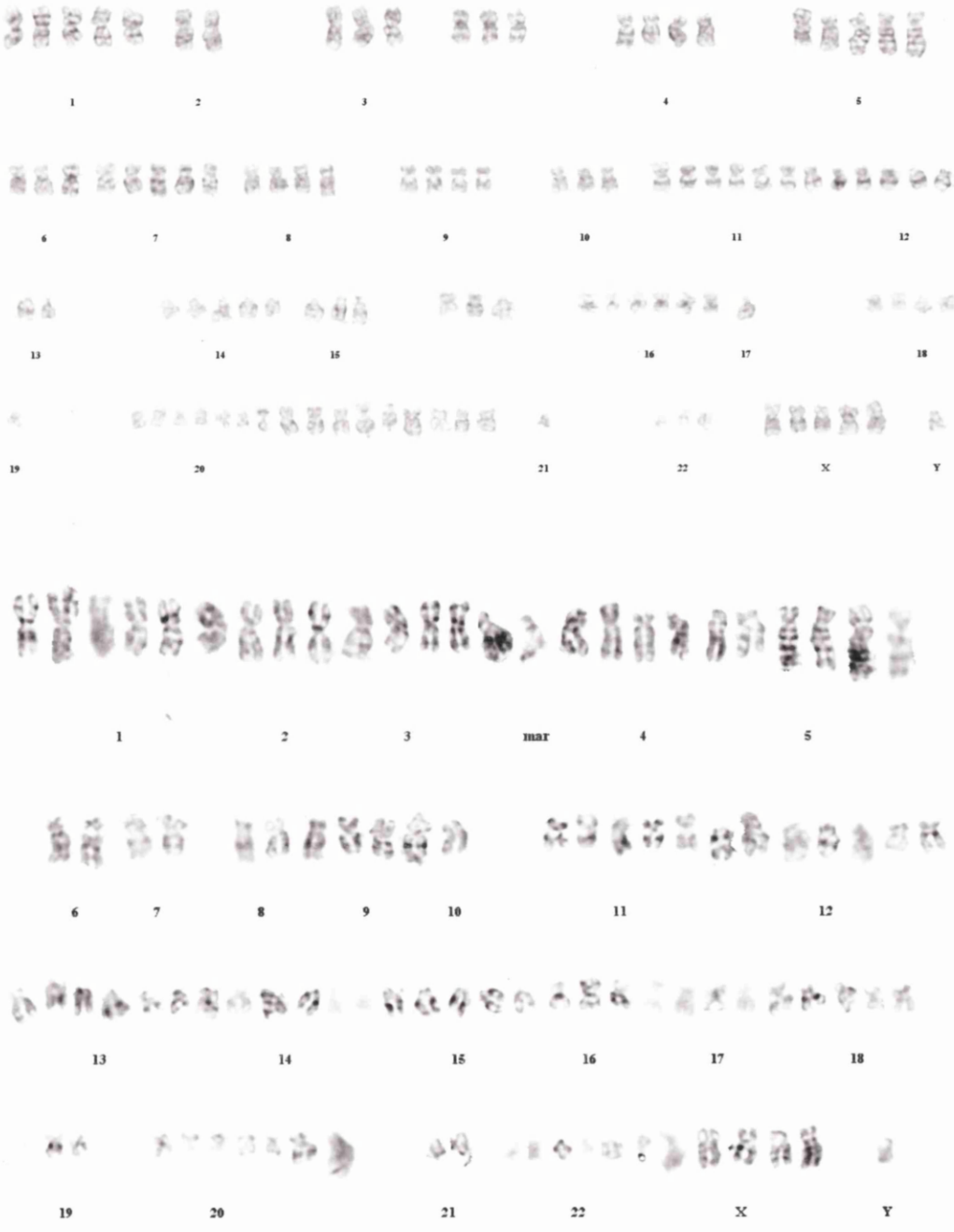


PLATE 3.2 Karyotyping of two different metaphase spreads of OE 33 cells using the G-banding technique. Picture demonstrates the difficulty in karyotyping due to gross aneuploidy and complex reaarangements of the chromosomes.

PLATES 3.3 to 3.15 show the different chromosomes that were analyzed using whole chromosome painting labeled with either biotin or FITC and detected with avidin-Texas Red or the FITC respectively.

PLATE 3.3 Shows the FISH analysis with whole chromosome probe for chromosome one (green) and chromosome sixteen (red). Most of the cells showed four copies of chromosome one and four copies of chromosome sixteen. None of the cells showed normal copies of either one or sixteen. Frequent deletions and translocations of both the chromosomes were also noted.

PLATE 3.4 shows FISH analysis of chromosome two (red) and chromosome seventeen (green). The cells in these metaphases revealed a modal population with three copies of chromosome two and four copies of chromosome seventeen. Two of the cells showed normal copies of chromosome two.

PLATE 3.5 shows FISH analysis of chromosome three (red) and chromosome fifteen (green). The probes revealed a modal cell population with three copies for both chromosome three and chromosome fifteen. None of the counted cells showed normal copies of either chromosome three or fifteen.

PLATE 3.3

Shows the whole chromosome painting of chromosome one (green) and chromosome sixteen (red). At least four copies of chromosome one and four copies of chromosome sixteen are seen with possible deletion and translocations.

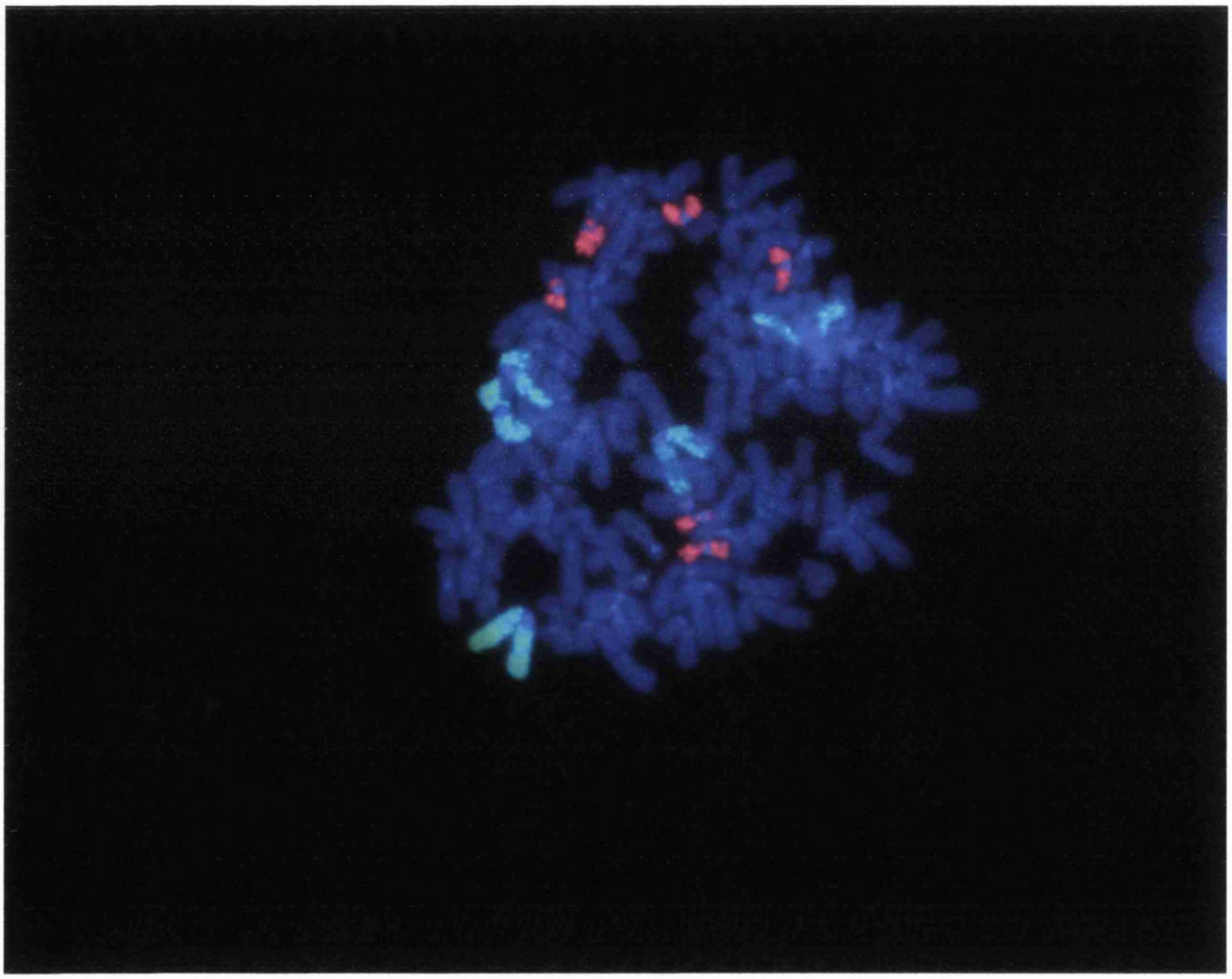


PLATE 3.4

Shows the whole chromosome painting of chromosome two (red) and chromosome seventeen (green). Multiple copies of chromosome seventeen and two copies of chromosome two are seen. Also seen in the picture is translocation of a fragment of chromosome seventeen (indicated by an arrow) on to an another chromosome.

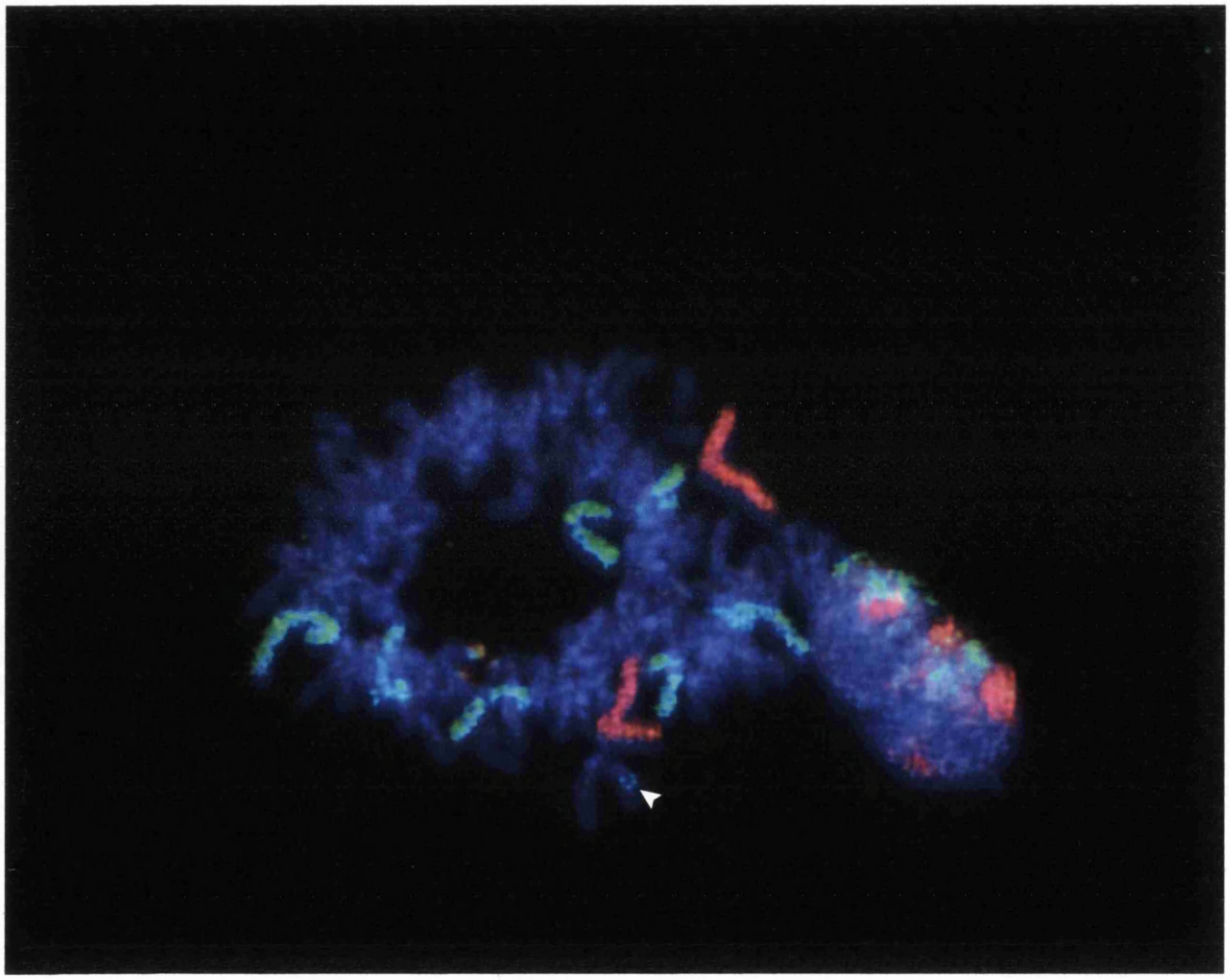


PLATE 3.5

Shows the whole chromosome painting of chromosome three (red) and chromosome fifteen (green). Three normal copies if chromosome three with a fourth copy with deletion (indicated by an arrow) are seen. There are two copies of chromosome fifteen with a deleted/translocated segment (indicated by an arrow).

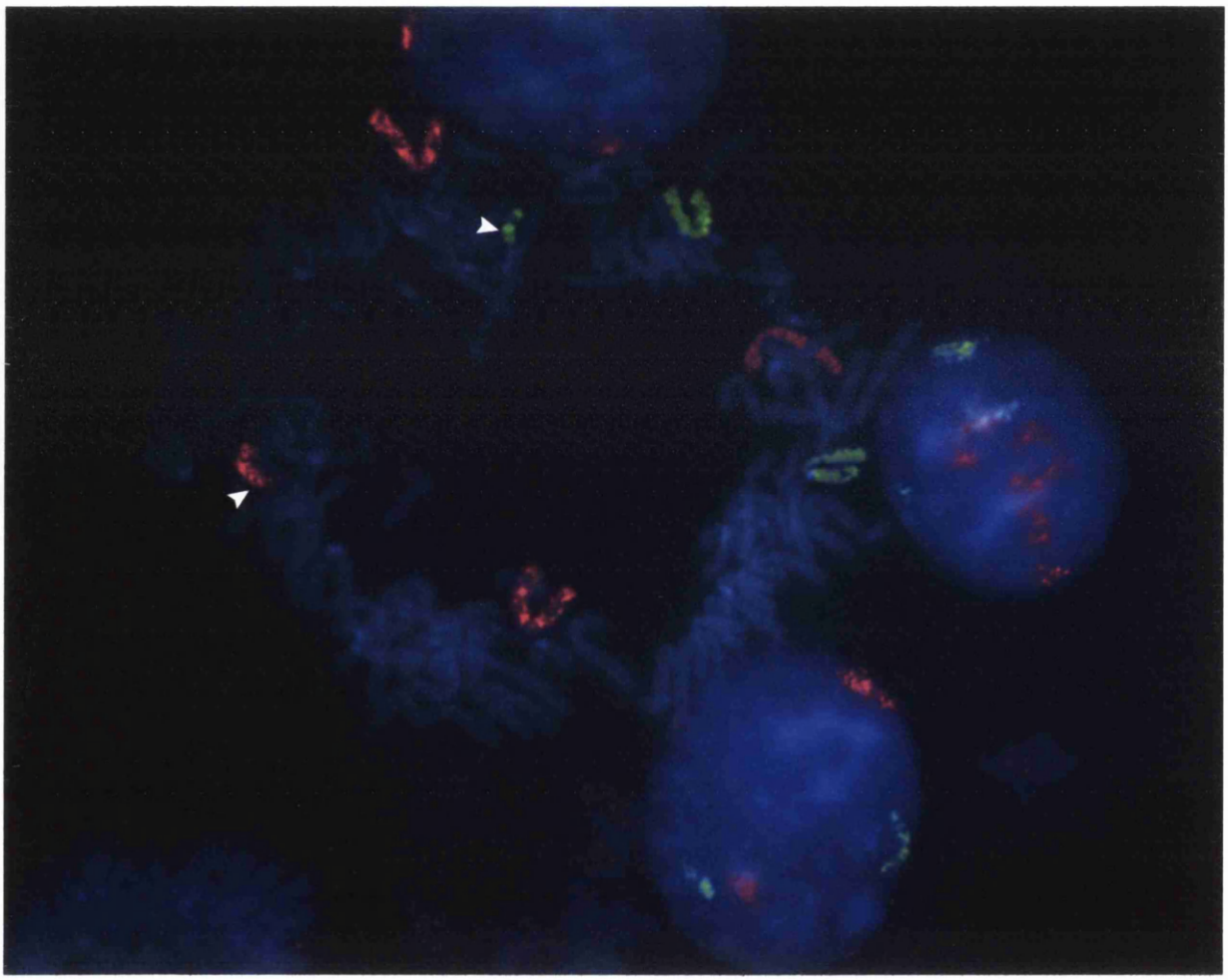


PLATE 3.6 shows FISH analysis of chromosome four (red) and chromosome twelve (green). The cells showed a modal population with four copies of chromosome four and three copies of chromosome twelve. None of the cells showed normal copies of chromosome four. Two cells showed normal copies of chromosome 12 whilst there was loss of one copy in one of the cells.

PLATE 3.7 shows the FISH analysis of chromosome five (green) and chromosome twenty (red). The cells showed a modal population with three copies of chromosome five and four copies of chromosome twenty. None of the cells had normal copies of chromosome twenty whilst two cells showed normal copies of chromosome five.

PLATE 3.8 shows the FISH analysis of chromosome six (green) and chromosome seventeen (red). Most cells showed three copies of both chromosome six and chromosome seventeen. Normal copies of chromosome six were seen in three of the cells.

PLATE 3.9 Shows the FISH analysis of chromosome eight (green) and chromosome four (red) using whole chromosome probes.

PLATE 3.10 Shows the FISH analysis of metaphase as well as interphase cell with probes for chromosome eight (green) and chromosome four (red). Most of the cells had predominantly four copies of chromosome four and chromosome eight. None of the cells had normal copies of either of the chromosomes.

PLATE 3.11 shows the FISH analysis of chromosome ten (green) and chromosome sixteen (red). The cells showed predominantly four copies of chromosome ten and three copies of chromosome sixteen. One of the cells showed loss of one copy of chromosome ten.

PLATE 3.6

Shows the whole chromosome painting of chromosome four (red) and chromosome twelve (green). Four copies of chromosome four and four copies of chromosome twelve are seen in the picture.

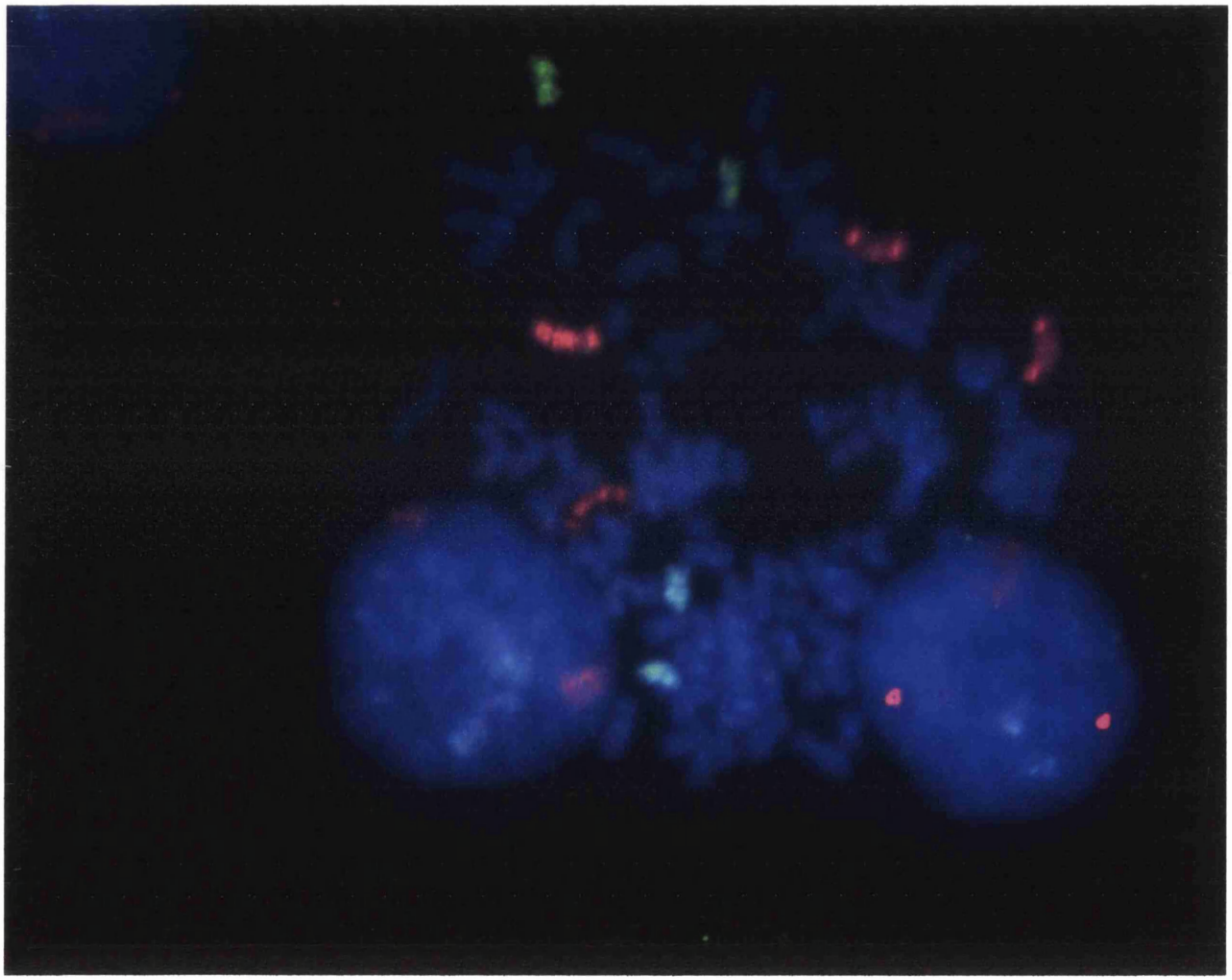


PLATE 3.7

Shows the whole chromosome painting of chromosome five (green) and chromosome twenty (red). Three copies of chromosome five with a translocated segment (indicated by an arrow) are seen. There are at least four copies of chromosome twenty

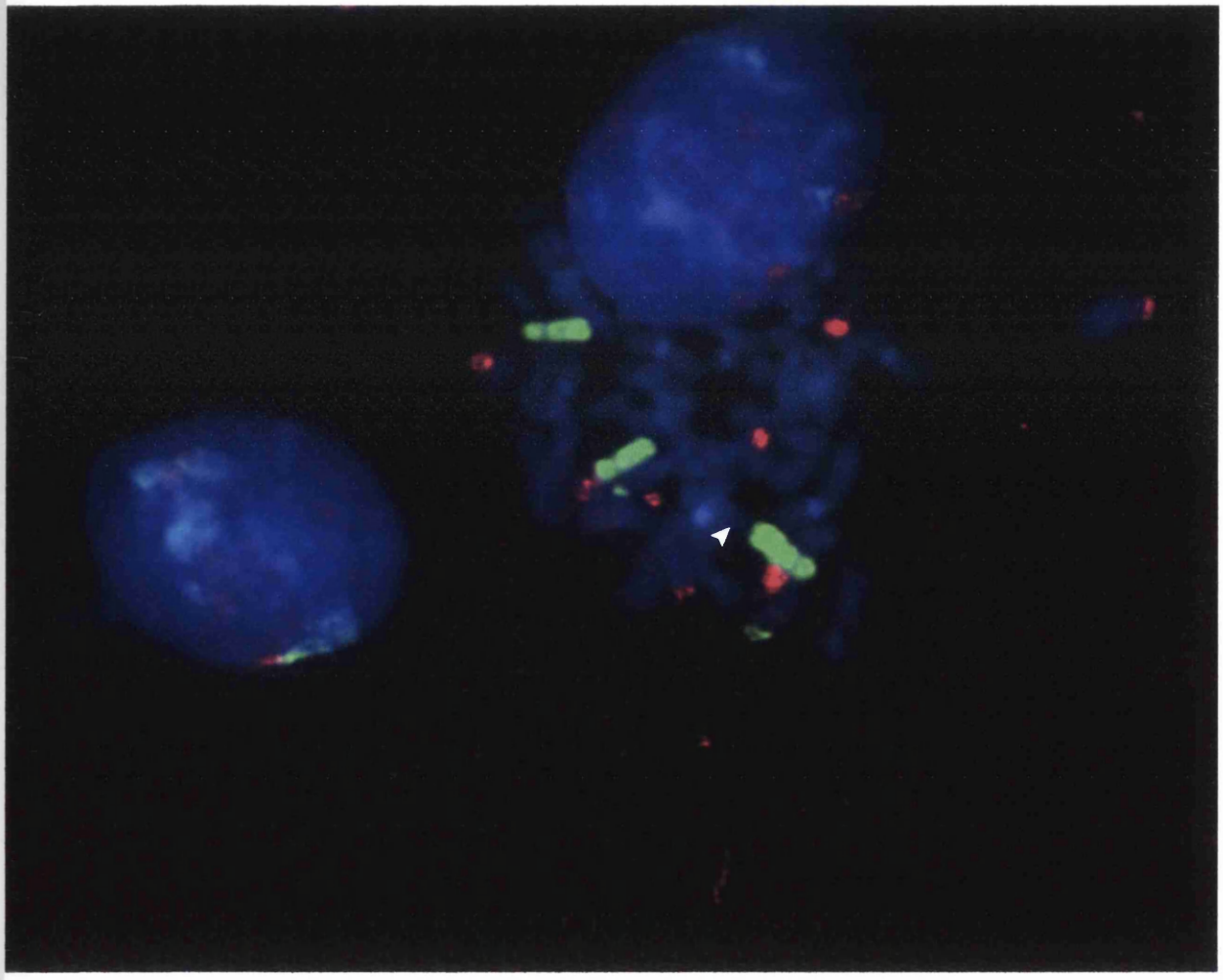


PLATE 3.8

Shows the whole chromosome painting of chromosome six (green) and chromosome seventeen(red). Two copies of copies of chromosome six are seen with a deletion and translocation (indicated by an arrow) are seen. There are at least three copies of chromosome seventeen.

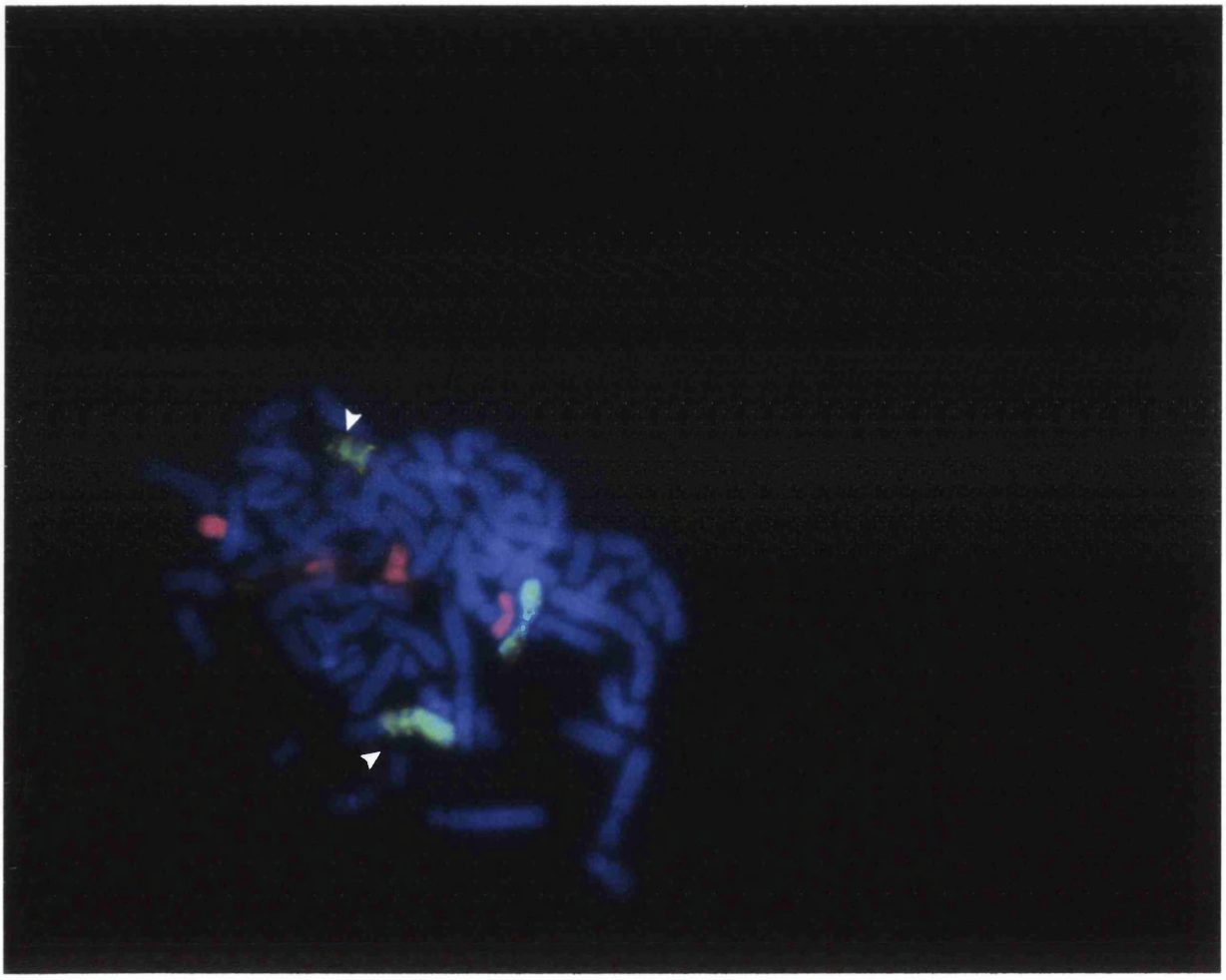
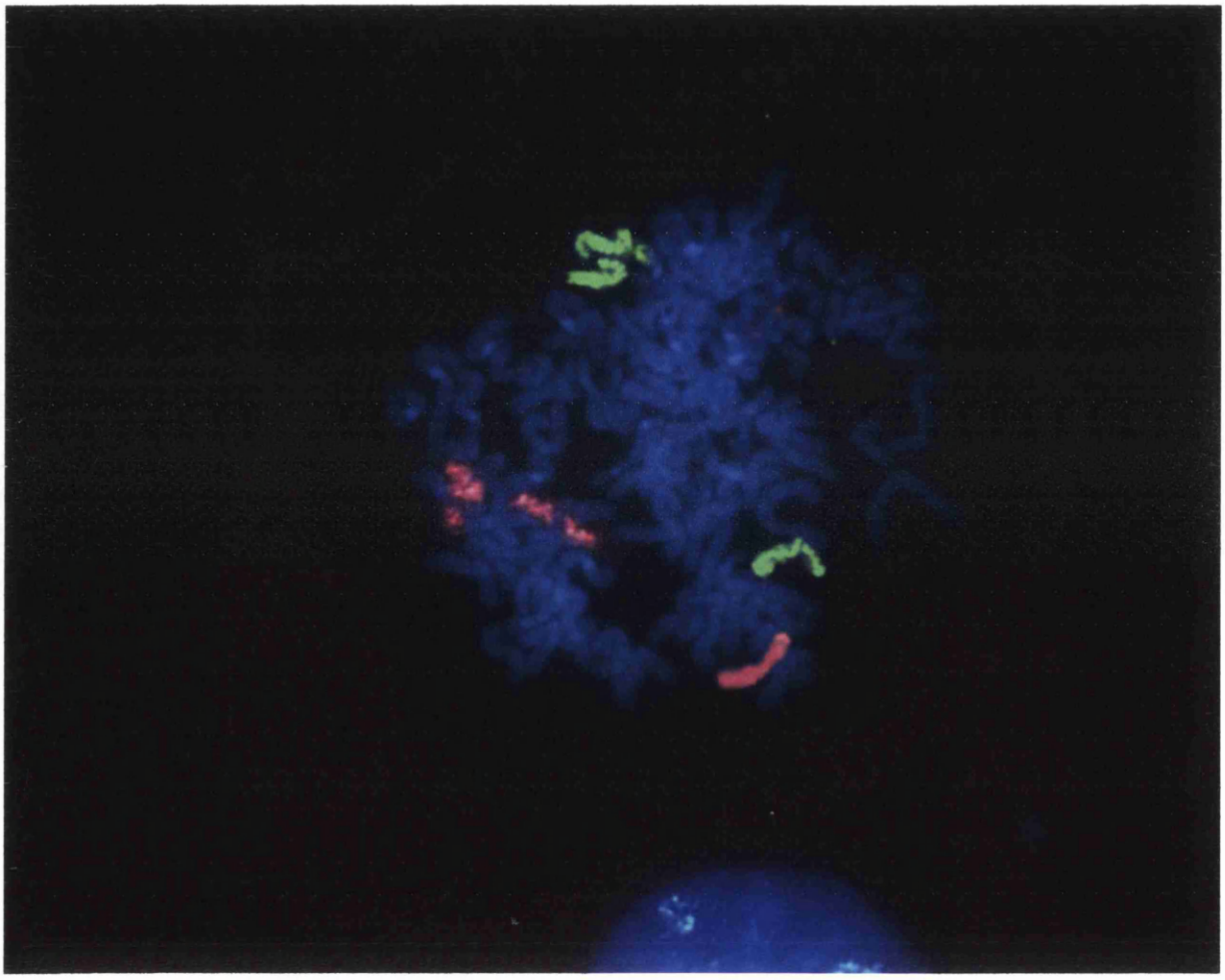
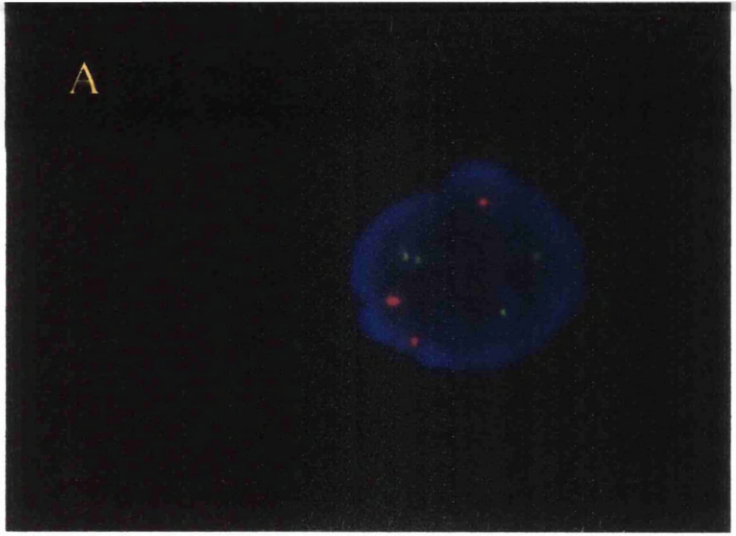
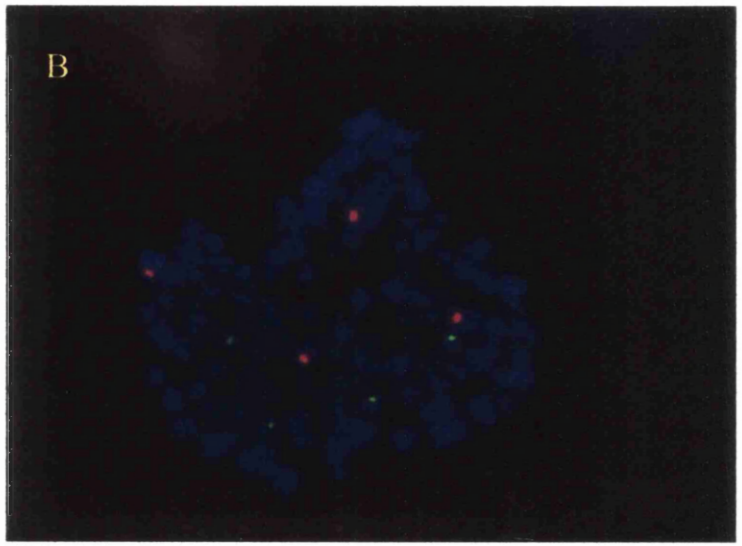


PLATE 3.9

Shows the whole chromosome painting of chromosome eight (green) and chromosome four(red). Three copies each of chromosome four and eight are seen in the picture.







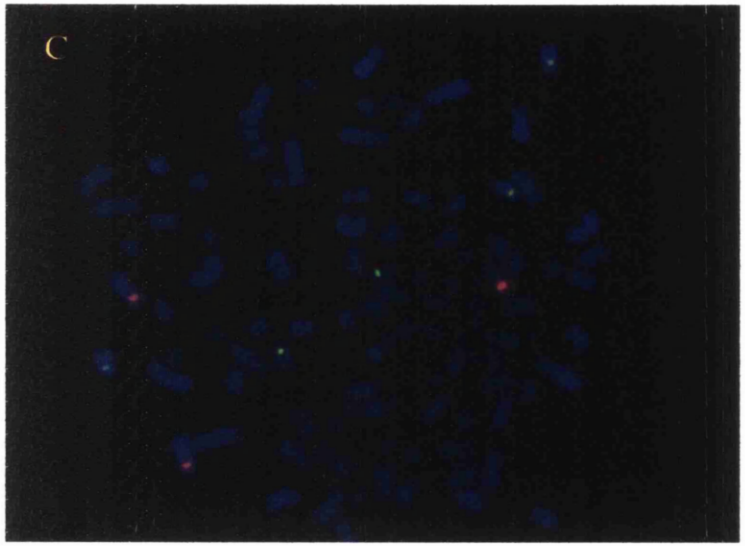


PLATE 3.10 Showing A. interphase slide with chromosome four (red) and chromosome eight (green) centromeric painting. B. & C. show metaphase FISH using centromeric probes for chromosome four (red) and chromosome eight (green) on OE33 cells.

PLATE 3.11

Shows the whole chromosome painting of chromosome ten (green) and chromosome sixteen (red). Four copies of chromosome ten and three copies of chromosome sixteen can be seen in the picture. Fragments of deletion/ translocation of chromosome sixteen can also be seen (as indicated by the arrow).

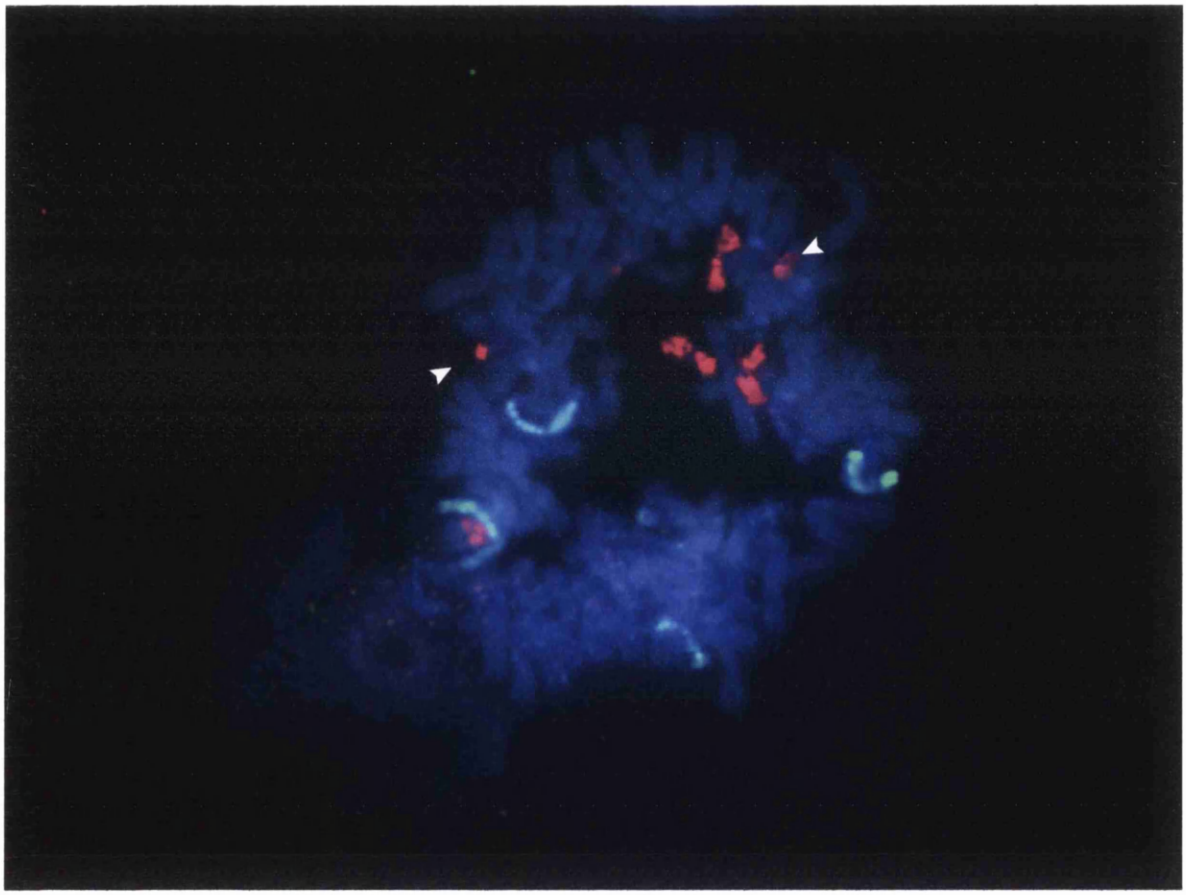


PLATE 3.12 shows the FISH analysis of chromosome eleven (green) and chromosome three (red). Most of the cells showed three copies of both chromosome eleven and chromosome three. In addition to this two of the cells had normal copies of chromosome eleven.

PLATE 3.13 shows the FISH analysis of chromosome seventeen (green) and chromosome four (red). The cells showed predominantly three copies of seventeen and four copies of four. There also loss of one copy of chromosome seventeen in two of the cells whilst two of the cells showed normal copies of chromosome seventeen.

PLATE 3.14 shows the FISH analysis of X chromosome (green) and chromosome twelve (red). The cell populations showed a modal three copies of X chromosome four copies of chromosome twelve. Normal copies of X chromosome were seen in two of the cells.

PLATE 3.15 Shows the FISH analysis of chromosome X and chromosome fourteen (green) and chromosome sixteen (red). Most of the cells showed three copies of both the chromosomes. Two of the cells showed normal copies of chromosome sixteen.

PLATE 3.12

Shows the whole chromosome painting of chromosome eleven (green) and chromosome three(red). Three copies each of chromosome three and eleven can be seen in the picture.

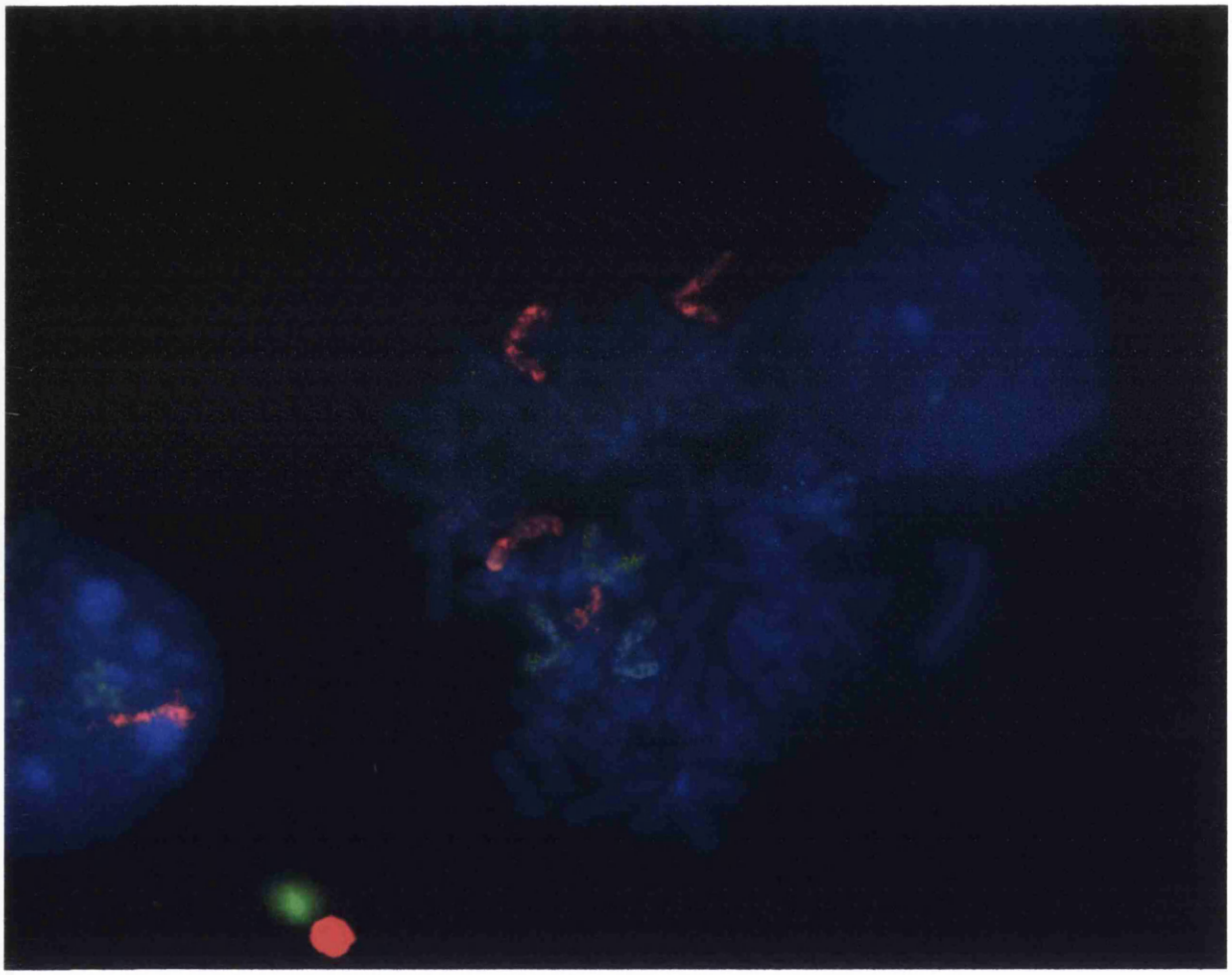


PLATE 3.13

Shows the whole chromosome painting of chromosome seventeen (green) and chromosome four (red). Three copies of seventeen and three copies of four with translocation to another chromosome can be seen (as indicated by the arrow).

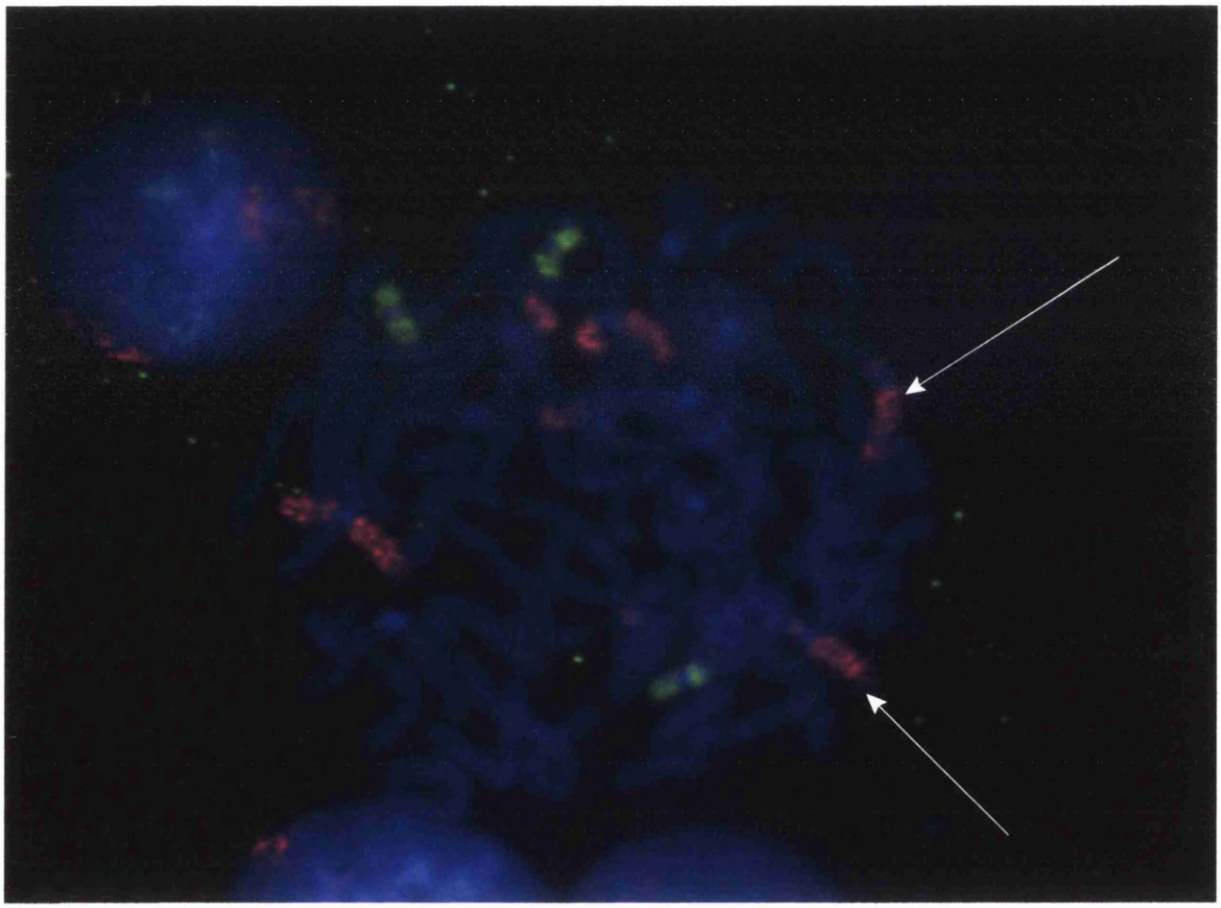


PLATE 3.14

Shows the whole chromosome painting of X chromosome (green) and chromosome twelve (red). Multiple copies of chromosome twelve (six) and at least three copies of Xchromosome are seen. Translocation of the X chromosome is also seen (as indicated by the arrow).

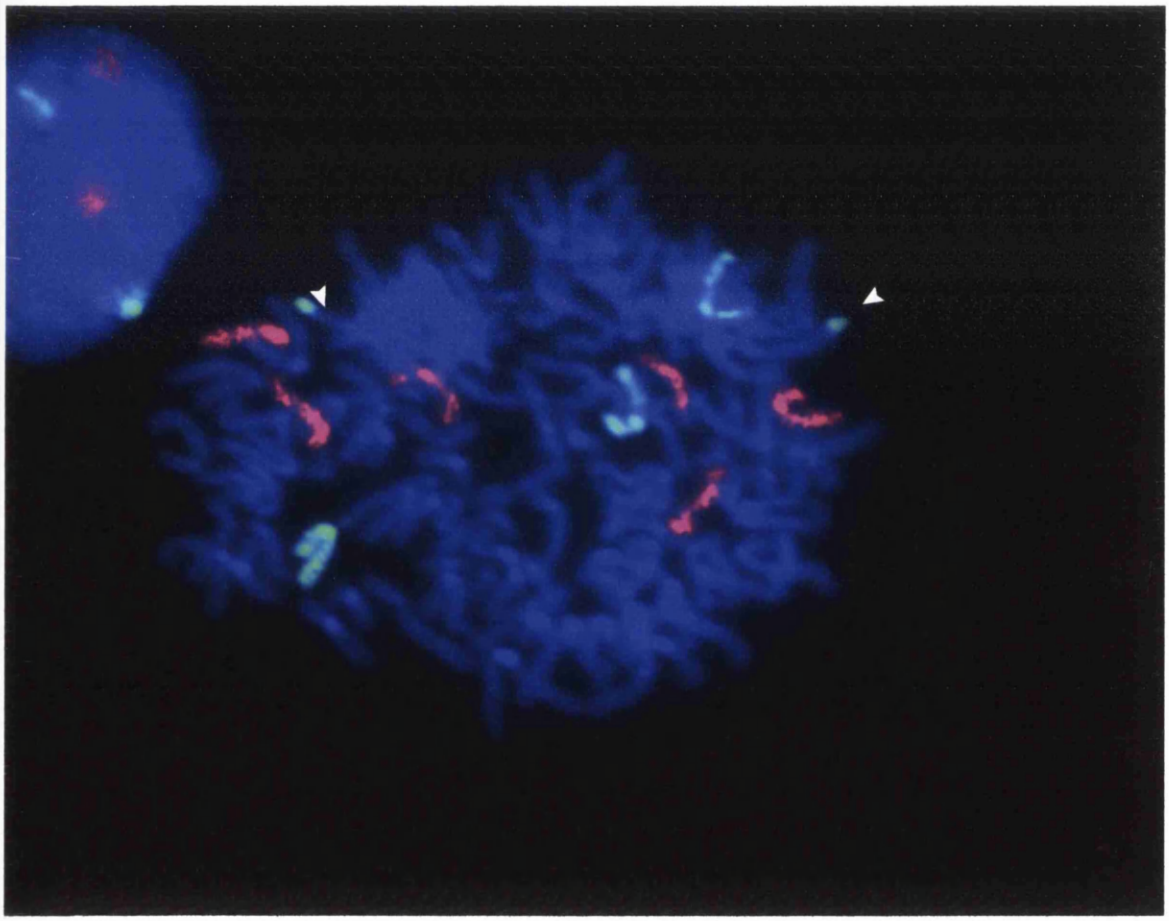


PLATE 3.15

Shows the whole chromosome painting of chromosome fourteen (green) and chromosome sixteen(red). More then three copies of both chromosome fourteen and sixteen are seen in the picture.

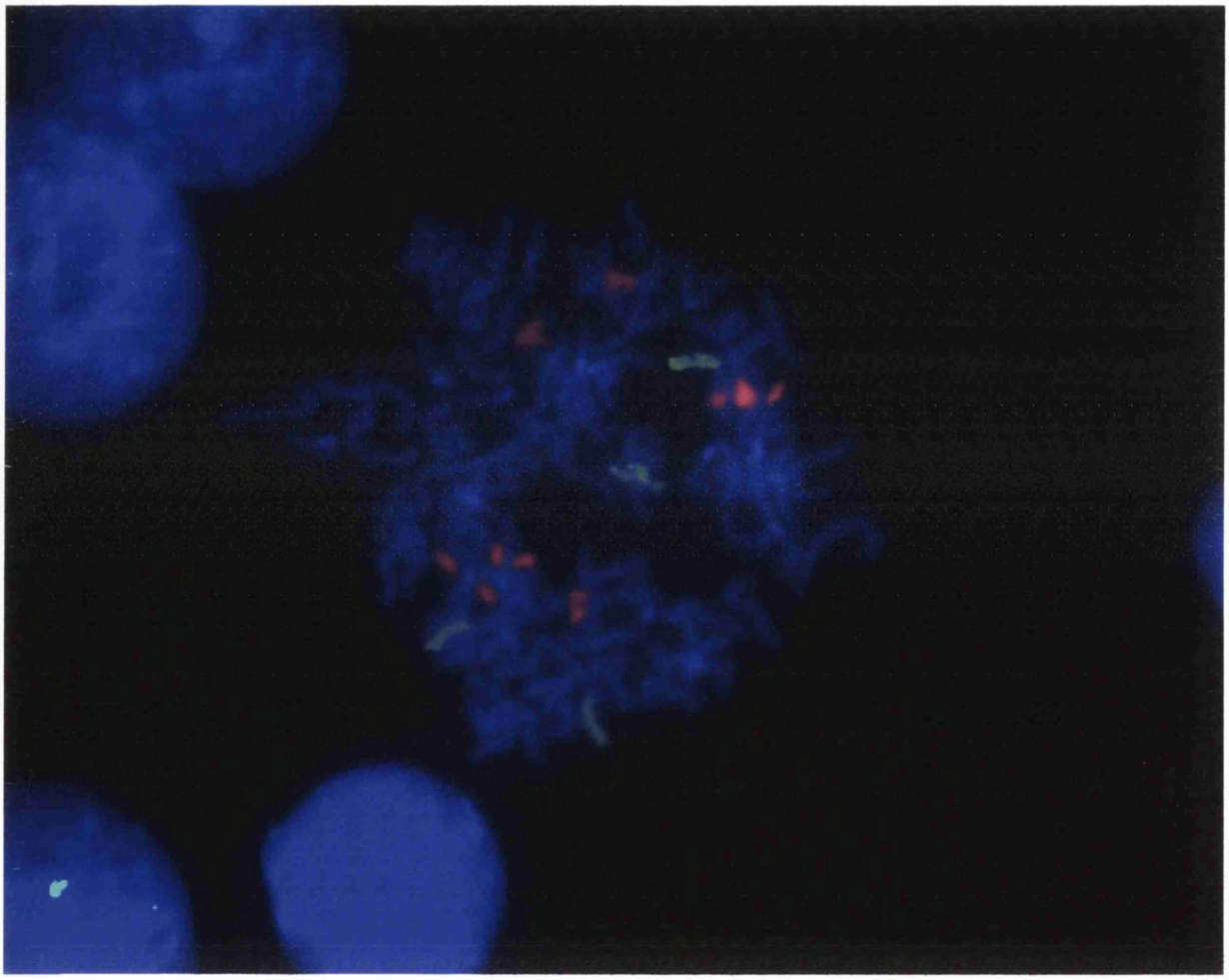


Table 3.1 Showing the results of the whole chromosome FISH analysis of the OE33 cells with probes for different chromosomes

CHROMOSOME	NO OF COPIES						TOTAL CELLSCOUNTED
	0	1	2	3	4	4+	
CHROMOSOME ONE AND SIXTEEN (SEE PLATE 3.3)							
CH ONE	0	0	0	7	10	3	20
CH SIXTEEN	0	0	0	6	12	2	20
CHROMOSOME TWO AND SEVENTEEN (SEE PLATE 3.4)							
CH TWO	0	0	2	10	7	1	20
CH SEVENTEEN	0	0	0	7	8	5	20
CHROMOSOME THREE AND FIFTEEN (SEE PLATE 3.5)							
CH THREE	0	0	0	6	11	1	18
CH FIFTEEN	0	0	0	5	9	4	18
CHROMOSOME FOUR AND TWELVE (SEE PLATE 3.6)							
CH FOUR	0	0	0	6	10	2	18
CH TWELVE	0	1	2	10	5	0	18
CHROMOSOME FIVE AND TWENTY (SEE PLATE 3.7)							
CH FIVE	0	0	2	12	4	2	20
CH TWENTY	0			4	11	5	20
CHROMOSOME (SEE PLATE 3.8)							
CH SIX	0	0	3	12	3	2	20
CH SEVENTEEN	0	0	0	14	4	2	20
CHROMOSOME FOUR AND EIGHT (SEE PLATE 3.9)							
CH FOUR	0	0	0	7	8	5	20
CH EIGHT	0	0	0	5	9	6	20
CHROMOSOME FOUR AND EIGHT (SEE PLATE 3.10) **CEP PROBE**							
CH FOUR	0	0	0	3	10	7	20
CH EIGHT	0	0	0	8	9	3	20
CHROMOSOME TEN AND SIXTEEN (SEE PLATE 3.11)							
CH TEN	0	1	0	4	9	3	17
CH SIXTEEN	0	0	0	8	6	3	17
CHROMOSOME ELEVEN AND THREE (SEE PLATE 3.12)							
CH ELEVEN	0	0	2	8	6	2	20
CH THREE	0	0	0	10	8	2	20
CHROMOSOME SEVENTEEN AND FOUR (SEE PLATE 3.13)							
CH SEVENTEEN	0	2	2	10	4	2	20
CH FOUR	0	0	0	8	10	2	20
CHROMOSOME X AND TWELVE (SEE PLATE 3.14)							
CH X	0	0	2	9	7	2	20
CH TWELVE	0	0	0	6	10	4	20
CHROMOSOME FOURTEEN AND SIXTEEN (SEE PLATE 3.15)							
CH FOURTEEN	0	1	0	12	3	1	17
CH SIXTEEN	0	0	2	10	2	3	17

In addition to the changes described above all the chromosomes showed frequent deletions and translocation which were impossible to characterize given the vast number of total abnormalities.

3.44 MICROTUBULE AND CENTROSOMES

The centrosomes can be visualized immunocytochemically with antibodies directed against γ tubulin (Oakley et al 1990).

γ tubulin is normally visualized as a dot on the nucleus or at the periphery of the nucleus. In the metaphase cells they are usually located at the two spindle poles on which the mitotic MTs spindles converge or nucleate. Although normal controls were not used (owing to inability to culture cells from normal oesophagus) in this experiment, most normal cells which are about to divide tend to contain one or two centrosomes per cell. The OE33 cells on the other hand the revealed gross abnormalities in both centrosome number and structure as revealed by γ tubulin staining. About 21% of the cells contained more than two centrosomes (table 3.2). They were heterogeneous in their shape and size. Large centrosomes, multiple centrosomes arranged in large patchy aggregates and centrosomes detached and lying outside the nucleus were frequently seen (plate 3.16). To demonstrate abnormalities in the spindle assembly during mitosis the cells were stained with γ tubulin. The OE33 cells showed abnormal spindles and spindles with poorly focused poles or multiple poles. Multipolar spindles were seen in about 17% of the cells (200 cells were counted) (plate 3.17). Some of the telophase cells showed abnormal multipolar divisions with the chromosomes segregating unequally into more than two daughter cells. These observations suggest that defects in centrosomes, spindle structure and chromosome segregation often occur together in cancer cells.

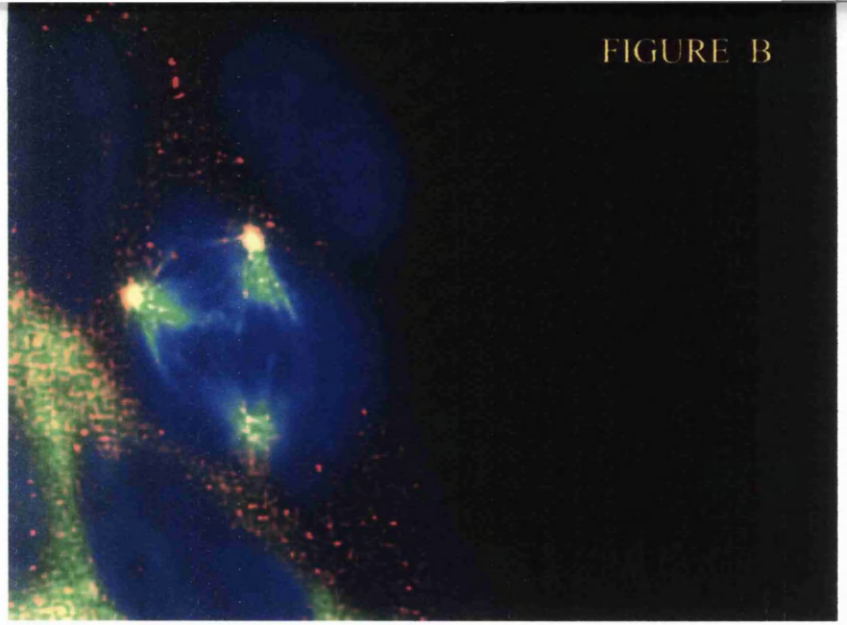
PLATE 3.16

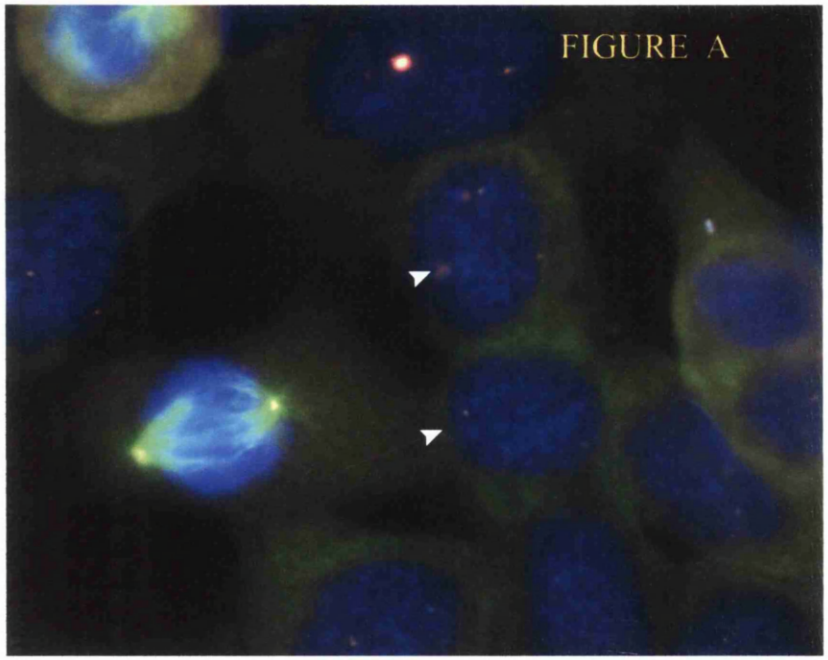
Shows microtubule staining of the OE33 cells

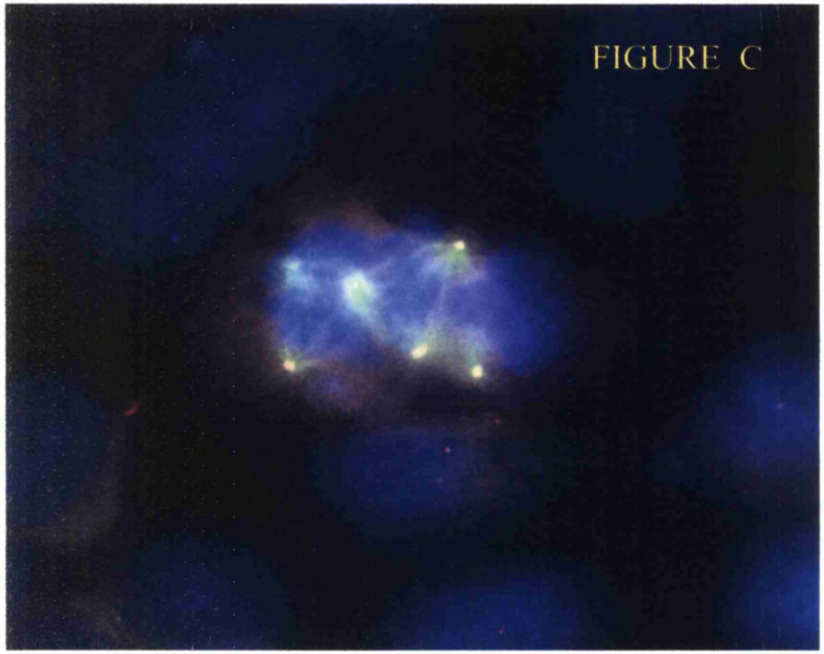
A. A mitotic cell with bipolar spindle. Arrows pointing at centrosomes seen as tiny dots overlapping the nuclei.

B. A mitotic cell with tripolar spindle

C. A mitotic OE33 cell with multipolar spindle.







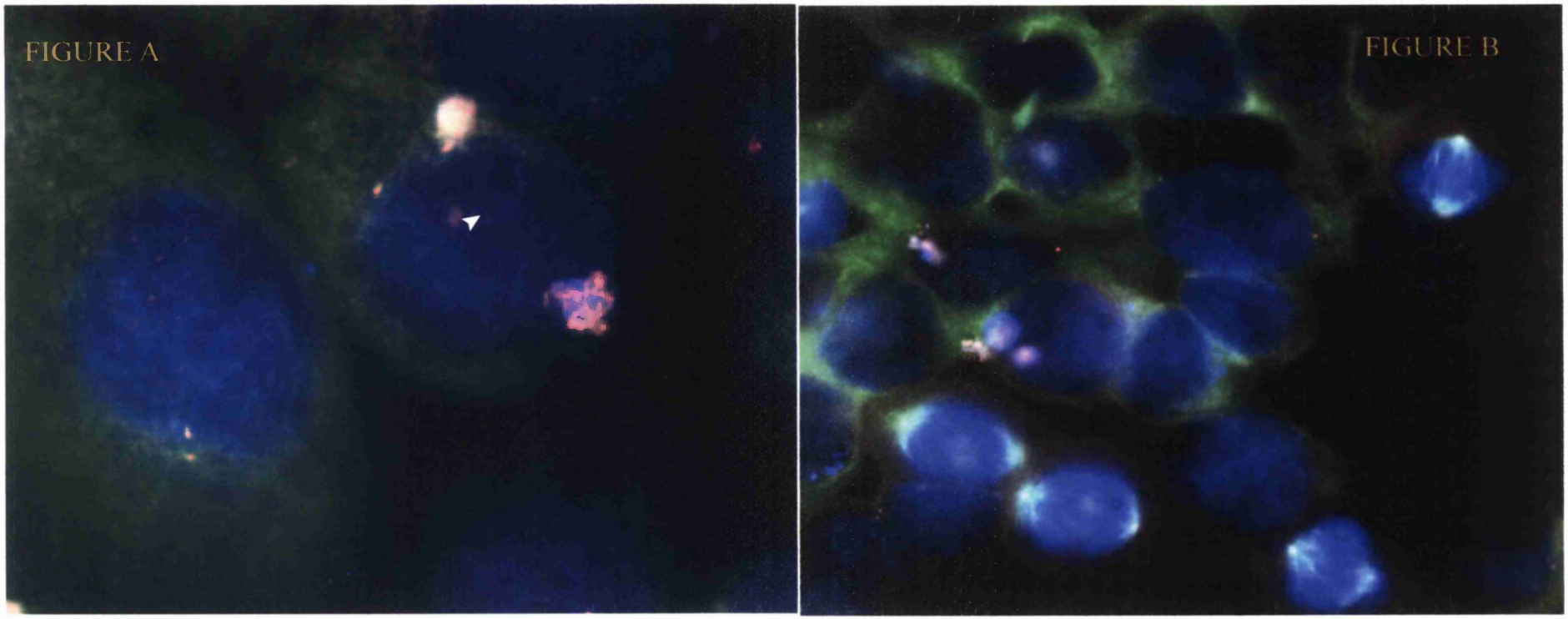


PLATE 3.17

A. shows multiple centrosomes organized into large patchy aggregates (arrow)

B. Shows mitotic cells with bipolar spindles and some large abnormal centrosomes

The centrosomes are tiny organelle composed of a pair of centrioles and surrounded by pericentiolar material (Rieder *et al.,* 2001). The centrosomes play a major role in the balanced chromosomal segregation during mitosis. Loss of centrosome function and regulation can lead to formation of abnormal spindles and chromosomal mis-seggregation. Structural and functional abnormality of centrosomes has been described in a number of different cancers (Pihan *et al.,* 1998). Aneuploidy which is a common feature in most cancers could be induced by a number of factors (discussed in chapter 4 in detail). Segregation errors leading to aneupoidy can be caused by mutation of mitotic check points or due to defects in cellular structures involved in chromosome segregation and mitosis. Owing to the major role played by the centrosomes in cell segregation, any defect in the structure and function of centrosomes could lead to aneuploidy. The centrosome abnormalities in our study did show a strong correlation with the degree of numerical chromosomal abnormality. This could partly explain many of the genetic changes and karyotypic abnormalities seen in the OE33 cells.

Table 3.2 Showing the results of centrosome and microtubule analysis

CELL LINE	ABNORMAL CENTROSOMES (%)	MULTIPOLAR SPINDLES		CHROMOSOMAL ABERRATION	
		Biaster (%)	Multiaster (%)	Numerical (%)	Structural (%)
OE 33	21	83	17	~ 100	~100

3.5 CONCLUSION

One of the hall marks of cancer cells is their genetic instability (Nowell 1976; Heim *et al.,* 1998). It is believed that cancer cells are monoclonal in origin (arise from a single

cell). Though most cancers are clonal they are found to be heterogeneous i.e., different chromosome patterns are found within individual tumors and among phenotypically similar tumors (Sandberg *et al.,* 1990; Mitelman F 1994; Heim S 1995; Johansson *et al.,* 1996; Mitelman F 1997). This heterogeneity could be in the form of alteration in the number and structure of the chromosomes. Cytogenetic analysis of the OE33 cells with G-banding and FISH has clearly demonstrated this fact. Studies have shown that cells lines undergo karyotypic changes with serial passages and have an increasing tendency to be polyploid. The OE 33 cells have a modal chromosomal number of 4n. Our cytogenetic analysis has shown that these cells have a high rate of ongoing structural and numerical chromosomal instability. Although there were several structurally altered chromosomes (or marker chromosomes) and centrosomal abnormalities, there was no common marker or unique chromosomal breakage common to all the cells. The high level of karyotypic complexity made the systematic characterization of the chromosome pattern difficult in this cell line.

The genetic instability of the cancer cells can be explained either by the genetic mutation theory (Nowell 1976) or the aneuploidy- genetic instability theory (Duesberg *et al.,* 1998). Studies have shown that the Karyotypic instability of the cell lines in vivo is directly proportional to the the degree of aneuploidy of the cell lines. As already observed (from both our studies and studies conducted by others) aneuploidy has been seen both in early and late stages of BE and OADC. Aneuploidy alone could explain genetic instability and the resulting karyotypic and phenotypic heterogeneity of cancer cells. The resulting heterogeneity in turn may permit selection and increase of aberrant cells that are responsible for tumour progression and metastasis.

CHAPTER FOUR

EVALUATION OF CYTOTOXICITY AND GENOTOXICITY OF THE BILE ACIDS

4.1 CHEMICAL CARCINOGENS

Carcinogenesis is a multi-step process. During this multi-step process a normal cell progressively acquires phenotypes which contribute to malignancy (Foulds 1954). The fact that cancer is caused by multiple genetic changes in a somatic cell is compatible with the view that many carcinogens induce mutation (Yamasaki *et al.*, 1995). It has been known for several hundreds of years that exposure to particular chemicals and mixtures can lead to cancers (Luch 2005). Paracelsus who described "wasting disease of miners" in 1567 was among the first to consider a chemical compound as an occupational carcinogen. In 1700 Ramazzini described the first example of occupational cancer. He noted that there was a high incidence of breast cancer in nuns, which he attributed to their celibate life. Later in the same century, the two English physicians John Hill and Percivall Pott described the occurrence of cancerous alterations in the nasal mucosa and at the skin of the scrotum in a few patients, and traced it to the local long-term exposure to snuff (Hill 1761) and to repetitive local contamination by soot, (Pott 1775) respectively. The Japanese pathologist Katsusaburo Yamagiwa and his assistant Koichi Ichikawa were the first to produce malignant epithelial tumours by application of coal tar to the ears of rabbits in 1915 (Yamagiwa 1915). The field of chemical mutagenesis has rapidly expanded and a great diversity of compounds have been discovered which are carcinogenic in humans.

The evidence to show that chemicals can damage DNA leading to mutation has been accumulating over the years (Bridges 1976; Russell *et al.,* 1985). The need to detect mutagenic chemicals has been well recognized due to the risk that these chemicals pose to the germ and somatic cells in man (Oshimura *et al.,* 1986).

4.12 CLASSIFICATION OF CHEMICAL CARCINOGENS

In an early approach chemical carcinogens were classified as initiators, promoters or complete carcinogens (Pitot 1986). This was based on the fact that the process of chemical carcinogenesis involved two stages, namely, initiation and promotion (Clayson *et al.,* 1991). Initiation is a relatively rapid and irreversible stage in which normal cells are converted to cells with the potential to become malignant. Promotion is a long lasting and partially reversible stage in which the initiated normal cells are encouraged to become tumours (Clayson *et al.,* 1991). However the distinction between these categories became increasingly blurred with increasing insight into the process of carcinogenesis (Butterworth *et al.,* 1995). A more useful and practical classification is the one suggested by Cohen and Ellwein which is based on the biological activity of the carcinogens (Cohen *et al.,* 1990). According to this classification the chemical carcinogens are divided into two broad categories; namely genotoxic and non-genotoxic carcinogens (see table 4.1). This distinction is of profound importance in developing short term tests (Clayson *et al.,* 1996).

4.121 GENOTOXIC CARCINOGENS

The term "genotoxic substances" was first used by Herman Druckrey in 1973. Druckrey defined genotoxic substances as "any agent which, by virtue of its physical or chemical properties, can induce or produce heritable changes in those parts of the genetic apparatus that exercise homeostatic control over somatic cells, thereby determining their malignant transformation" (Weisburger 1999). Genotoxic (or

genotoxicity) refers to agents which interact with the DNA and/or the cellular apparatus which regulates the fidelity of the genome, e.g. the spindle apparatus, and enzymes such as the topoisomerases. It is a broad term that includes mutation as well as damage to DNA or the production of DNA adducts, by the chemical itself or its metabolites (Parry 2000).

TABLE 4.1: Classification chemical carcinogens (Adapted from Butterworth *et al.*, 1995).

CLASSIFICATION OF CARCINOGENS ACCORDING TO MODE OF ACTION
GENOTOXIC CARCINOGENS
DNA reactive or DNA reactive metabolites Direct interaction to alter chromosome structure or number May also be cytotoxic Regenerative cell proliferation will enhance mutagenic/carcinogenic activity. May also be mitogenic Induced cell proliferation will enhance mutagenic/carcinogenic property.
NON-GENOTOXIC CARCINOGENS
CYTOTOXICANTS Not DNA reactive Cytolethal at carcinogenic Doses Induce regenerative growth Mutations may occur secondary to regenerative cell proliferation Accompanying critical effect may occur as inflammation. Circulating growth factors may cause preferential growth of pre-neoplastic cells
MITOGENS Not DNA reactive Not cytolethal at carcinogenic doses Mitogenic stimulation of growth May be acting through a specific receptor Mutations occur secondary to cell proliferation May stimulate preferential growth of pre-neoplastic cells

Chemical carcinogens can induce mutation at three different levels (Bridges *et al.*, 1989) namely,

1. At the gene level
2. At the individual chromosome level (Clastogenicity)
3. At the level of chromosomal set (Aneuploidy)

Clearly information at these three different levels of mutation, namely gene, clastogenicity (i.e. structural chromosome aberrations) and aneuploidy (i.e. numerical chromosomal aberrations), is necessary to provide comprehensive coverage of the mutagenic potential of a substance (Parry *et al.,* 2002). This is also required for assessing the carcinogenic potential of a substance, since all three types of mutation have been shown to be associated with the activation and expression of oncogenes, and loss or inactivation of tumour suppressor genes and other classes of genes implicated in carcinogenesis (Parry *et al.,* 2002).

4.1211 GENE MUTATION

Mutations involving single genes may be a consequence of effects on single DNA bases (point mutations) or of larger changes, including deletions, insertions and rearrangements of DNA (Pihan *et al.,* 2003). Point mutations can be in the form of transitions, transversions or frameshift mutations. The gene mutation assays are of two categories, those which detect forward mutations and those which detect reversion (Parry 2000). Forward mutations alter a wild type gene, and inactivation of the gene results in a detectable change in the phenotype. A reversion on the other hand restores gene function in a mutant and thus brings about a return to the wild phenotype. The Ames test is a good example for reverse mutation assay. The test uses strains of salamonella *typhimurium* which cannot synthesis amino acid histidine and therefore will fail to grow in histidine deficient medium. However a reversion of

mutation (due to exposure to a mutagen) restores the functional capability of the bacteria to synthesise histidine, therby allowing the bacteria to grow in colonies in histidine deficient medium (Mortelmans *et al.,* 2000).

The mouse lymphoma assay (MLA) is a good example for forward mutation assay. The MLA assay uses the L5178Y *tk*+/- mouse lymphoma cells which have one functional copy of the gene that codes for thymidine kinase (TK). Forward mutation of this cells (from *tk*+/- to $tk^{-/-}$) leads to formation of mutant cells deficient in TK. Cells deficient in TK are resistant to the cytotoxic effect of pyrimidine analogues such as trifluorothymidine (TFT) and therefore survive and form colonies, while cells without mutation have their growth totally arrested. The mutagenicity of the test agents is indicated by the increase in the number of colonies after treatment (Clements 2000).

4.1212 CHROMOSOMAL ABERRATIONS

Damage to individual chromosome leads to chromosomal breakage. This could be in the form of translocations (reciprocal translocation, Robertsonian translocation) inversions or deletions (Bridges 1989). An agent, which induces chromosomal aberration, is called clastogen (Oshimura *et al.,* 1986). Structural chromosomal aberrations result from A. direct DNA breakage; B. replication on a damaged DNA template; C inhibition of DNA synthesis and other mechanisms (e.g., topoisomerase-II inhibitors) (Aardema *et al.,* 1998). Structural and numerical chromosomal aberrations play an important role in carcinogenesis (Tucker *et al.,* 1996; Duesberg *et al.,* 1998). Increased frequency of structural chromosomal aberrations in peripheral blood lymphocytes has been shown to be associated with an increased overall risk for cancer.

Chromosome aberrations involve gross alteration of genetic material which can be detected by light microscopy in a metaphase spread. Analysis of chromosomal

aberration is mostly performed in the CHO cells or cultured lymphocytes. Structural and numerical chromosomal aberrations are most commonly scored in proliferating cells arrested at metaphase using a tubulin polymerisation inhibitor e.g. Colcemid, colchicines. Chromosomal aberrations can also be detected in metaphase or interphase-cells using fluorescence in-situ hybridisation FISH. This technique provides increased efficiency and specificity for identifying certain kinds of chromosomal aberrations (i.e. chromosome-type structural rearrangements, stable symmetrical rearrangements derived from chromatid-type chromosome aberrations, hyperploidy) induced in-vivo. However, the drawback with FISH technique is that only hyperploid events are scored due to the potential for technical artifacts resulting in the loss of a hybridisation signal (Aardema *et al.,* 1998).

The evaluation of chromosome aberration by classical metaphase preparations is time consuming and labour intensive. An easier and equally efficient method to detect clastogens and aneugens is the in-vitro micronucleus assay. The in-vitro micronucleus test is the only test which is able to detect both numerical and structural chromosomal aberrations (Marzin 1997). In fact it has been proposed as an alternative to the metaphase analysis for the biological risk assessment of chemicals (Miller *et al.,* 1995). The in-vitro micronucleus test will be described in detail further in this chapter.

4.1213 ANEUPLOIDY

The third type of mutation which involves the chromosomal set is aneuploidy. Aneuploidy refers to change in chromosome number from the normal diploid or haploid number for the species (Aardema *et al.,* 1998). Of the different classes of chromosome abnormality, aneuploidy is by far the most common and, clinically, the most important (Hassold *et al.,* 1996). Aneuploidy in germ cells leads to spontaneous abortions, infant deaths and congenital malformations (Hassold *et al.,* 1996). Aneuploidy in somatic cells is associated with the development of several cancers

(Fearon *et al.,* 1990; Cavenee *et al.,* 1991). Aneuploidy has been seen in both early and late stages of cancer (Aardema *et al.*, 1998). In fact many believe that aneuploidy could be the somatic mutation that causes cancer (Brinkley *et al.,* 1998; Duesberg *et al.,* 1998; Duesberg *et al.,* 1999; Rasnick *et al.,* 1999; Li *et al.,* 2000). Studies have shown that a large number of aneugens are inducers of malignant transformation in Syrian hamster cells *in-vitro* ((Oshimura *et al.,* 1986; Parry *et al.,* 1993; Gibson *et al.,* 1995). Aneuploidy can result from damage to a variety of structures such as tubulins, centromeres/kinetocores, centrioles/centrosomes, microtubule associated proteins and regulatory molecules (Liang *et al.,* 1985; Oshimura *et al.,* 1986). The different mechanisms by which chemicals can induce aneuploidy are summarized in table 4.2. There are a number of methods to detect the induction of aneuploidy. Some assays are restricted to specific targets, such as mitotic spindle in an assay for effects on the polymerisation of tubulin in-vitro (Parry JM 1993).

Table 4.2: MECHANISMS BY WHICH CHEMICALS CAN INDUCE ANEUPLOIDY (Adapted from Oshimura *et al.*, 1986)

Damage to essential elements for chromosome function (centromeres, origins of replication, and telomeres)
Reduction in chromosome pairing
Induction in chromosome interchanges
Effects on chromosome condensation
Persistence of the nucleolus in mitosis or meiosis
Increased chromosome stickiness
Effects on microtubules
Damage to centrioles or kinetochores
Impairment of chromosome alignment
Alterations in ion concentration in mitosis
Damage to nuclear membrane
Physical disruption of chromosome segregation

Others, such as chromosome counting in a metaphase spread, detection of micronuclei that contain kinetochores (Natarajan *et al.,* 1993) and gross examination for abnormal spindles and spindle-chromosome associations in cells in which spindles and

chromosomes have been differently stained (Warr *et al.,* 1993) tend to detect aneuploidy itself.

Chromosomes can be studied directly by observing and counting aberrations in metaphases (Natarajan *et al.,* 1982). This approach provides the most detailed analysis, but the major drawback is complexity and laboriousness of enumerating aberrations in metaphase and the confounding effect of artefactual loss of chromosomes from metaphase preparations.

The major drawback of counting chromosomes in a metaphase spread is that it is time consuming and limited to evaluation of hyperploidy because decreases are frequently due to artefacts of slide preparation. As described earlier in this section, this problem can be overcome by using the FISH technique and the in-vitro micronucleus assay (Tucker *et al.,* 1996).

4.122 NON GENOTOXIC CHEMICALS

As already described, non-genotoxic compounds are those that lack DNA reactivity or direct chromosome altering activity as a primary biological effect (Butterworth *et al.,* 1995). Unlike genotoxic substances which can be detected by a range of in-vitro assays, non-genotoxic chemical detection depends principally upon 2 year rodent bioassays. Molecular targets of non-genotoxic carcinogens can include all cellular and extra-cellular constituents of various organs, except the DNA. Owing to the diversity of their mechanism of action it is difficult to define one or two endpoints by which non-genotoxic carcinogenesis can be studied (Yamasaki 1995). Epigenetic carcinogens can induce transformation by increasing intracellular reactive species (reactive oxygen species, nitric oxide and lipid peroxidation products) that have a potential to modify DNA, by stimulation of cell proliferation (can enhance spontaneous mutations) or by inhibition of apoptosis (Williams 2001). Various markers have been used as a basis of tests for non-genotoxic carcinogens. These

include inhibition of intercellular gap junctional intercellular communications, inhibition of tubulin polymerisation, and inhibition of apoptosis and induction of cell proliferation (Combes 2000). Most risk assessments however use genotoxicity as a qualitative hazard because most human carcinogens belong to this category (Williams *et al.,* 1988; Weisburger 1999). However, many of the mutagenic agents also have non-mutagenic epigenetic mechanisms which are important to their carcinogenic activity. One such example is nickel. The mechanism of nickel induced carcinogenesis is likely to involve both genetic and epigenetic routes. High concentrations of nickel can induce DNA damage by generation of reactive oxygen species (ROS) and inhibiting DNA repair. The epigenetic effects of nickel include alteration gene expression resulting from DNA hypermethylation and histone hypoacetylation, as well as activation or silencing of certain genes and transcription factors, especially those involved in cellular response to hypoxia (Kasprzak *et al.,* 2003).

4.14 THE MICRONUCLEUS ASSAY

4.121 HISTORICAL ASPECTS OF MICRONUCLEUS ASSAYS

The description of micronuclei dates back to as early as 1930s and 1940s. Induction of micronuclei was observed by a number of different cytogeneticists who were studying the effects of x-ray irradiation on diverse materials, such as grasshopper neuroblasts, onion root tip meristems (Sax 1941) and tradescantia pollen grains (Koller 1943). The micronucleus assay was used for the first time in-vitro in radiation experiments with roots of *vicia faba* by Evans *et al* in 1959 (Evans *et al.,* 1959). This was the first realistic attempt to use micronucleus assay to measure the chromosome damaging effects of mutagenic agents. Later on in 1973 Schmid *et al* and Heddle independently described the use of micronucleus assay in vivo experiments, for the first time for detection of chromosome damage, induced in animals following exposure to

mutagens (Heddle 1973; Schmid 1973). A large number of studies have been conducted since, both in-vitro and in-vivo using a variety of mutagens and a range of different cell types.

In the original method proposed by Countryman and Heddle (Countryman *et al.,* 1976) MN were scored in dividing cells without discriminating between cells that had completed nuclear division and those that had completed nuclear division following exposure to the mutagen. It was realised that this method under estimated the frequency of MN when nuclear division was inhibited or when the cells divided more than once (Fenech *et al.,* 1985). In the former case the MN frequency is reduced because MN are formed only in dividing cells and in the latter case the MN frequency is reduced because of the loss of MN from micro-nucleated cells and/or dilution of micro-nucleated cells that divide once by non-micro-nucleated cells that have divided more than once (Fenech 2000). This major problem was however overcome with the introduction of cytokinesis blocked micronucleus assay by Michael Fenech and Alec Morley in 1985. They used Cytochlasin B to inhibit cytokinesis without blocking mitosis. This enabled them to score MN in cells which had divided once only. Since then the MN assay has been adopted worldwide as a sensitive and reliable method for assessing chromosomal damage.

4.122 DEFINITION

Micronuclei are chromatin containing structures in the cytoplasm surrounded by a membrane without any detectable link to the cell nucleus (Schiffmann *et al.,* 1991). Micronuclei are formed in actively dividing cells. These micronuclei originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes which are unable to migrate to spindle poles during mitosis. These lagging chromosome and fragments acquire a nuclear envelope during telophase and assume the morphology of a nucleus, except that they are smaller in size than the

actual nucleus (Fenech 1997). In the cytokinesis blocked MN assay cells which have completed only one nuclear division are counted.

4.123 CYTOKINESIS BLOCKED MN ASSAY; A MULTI ENDPOINT ASSAY

The CBMN assay is a multi-endpoint assay which enables simultaneously to detect mitotic delay, necrosis, apoptosis, chromosome loss, chromosome breakage and non-disjunction (Kirsch-Volders M 1997). It provides information on cell cycle progression and chromosome / genome mutation at the same time. Thus, MN provide a measure of both chromosome breakage and chromosome loss and it has been shown to be as sensitive an indicator of chromosome damage as classical metaphase chromosome analysis (Matsuoka *et al.,* 1993; Miller *et al.,* 1995).

The key advantage of the MN assay is

1. The relative ease of scoring and the statistical power obtained from scoring larger numbers of cells than are typically used for metaphase analysis.
2. Frequencies of cytokinesis blocked bi-nucleated cells (and poly-nucleated cells) are considered as reliable estimators of the mitotic rate.
3. Based on morphological criterias necrotic and apoptotic cells can be differentiated from viable cells.
4. Combination of micronucleus scoring and FISH with centromeric, pericentromeric or telomeric probes allows the discrimination between aneugens and clastogens.
5. Use of appropriate FISH probes also enables to distinguish between chromosome loss and non-dysjunction.

4.124 DETECTION OF ANEUGENS AND CLASTOGENS

There are various ways by which an aneugen can be distinguished from clastogen in the in vitro micronucleus assay.

A quick, simple, inexpensive but less reliable way of detection is based on morphology of the micronuclei (Matsuoka *et al.,* 1999). A number of studies have shown that the size and morphology of the micronucleus can be used to distinguish an aneugen from clastogen (Yamamoto *et al.,* 1980; Hogstedt *et al.,* 1985). It has been observed that the relationship between an average chromosome and an average fragment is 4:1. Hence a larger micronucleus is suggestive of a whole chromosome rather than a fragment (Kirsch-Volders 1997). This can however lead to erroneous results because large MN may contain more than one acentric fragment. Acentric fragments of a large chromosome may have a size and DNA content exceeding that of MN containing entire small chromosomes and DNA replication and hence, increase of DNA content may occur in MN (Schuler *et al.,* 1997). Matsuoka *et al* suggested a method to distinguish aneugen from clastogen based on the frequency of polynuclear cells (PN) and mitotic cells (M). They found that aneugens induced more than 200 PN cells/1000 cells while clastogens usually induced less than 100 PN cells/1000 cells. The aneugens were found to induce a higher frequency of M cells (Matsuoka *et al.,* 1999). A high ratio of large MN to total micronucleated cells is more suggestive of an aneugen than clastogen.

A more reliable technique is the use of immunofluorscent staining with anti-kinetochore antibody (Eastmond *et al.,* 1989; Fenech *et al.,* 1989). This technique was first employed by Vig and Swearngin in 1986. They used serum from CREST patients (Calcinosis, Raynaud phenomenon, Oesophageal dysmotility, sclerodactyly and telangiectasia) to detect the kinetochores. The CREST serum contains antibodies to a specific protein of the kinetochore region of the chromosomes (Kirsch-Volders 1997). The centromere kinetochore complex is located at the primary constriction of the mitotic mammalian chromosomes and is responsible for the proper segregation of the replicated sister chromatids to the daughter cells (Warburton 2001). Kinetochores

form when specialized proteins bind to specific centromeric DNA sequences (Ault *et al.,* 1994). The kinetochore of higher eukaryotes usually appears as a tri-laminar structure (see figure 4.1) consisting of a well defined outer plate that is separated from, but connected to, a poorly defined inner plate intimately associated with the chromosome (Ault *et al.,* 1994). The antibodies in the CREST serum bind to kinetochores of mammalian cells in general. One of the major disadvantages with kinetochore staining is that, it visualizes the kinetochore proteins rather than the centromeric DNA itself. Hence aneuploidy induced by an agent (eg: Mitomycin c and 5-azacytidine) which inactivate or inhibit the formation of kinetochore proteins cannot be visualised (Kirsch-Volders 1997). With the advances in in-situ hybridisation technique more accurate methods have been developed to distinguish a clastogen from an aneugen. Centromeric probes can be used to accurately detect the whole chromosomes. The centromeric probe detects highly repeated *alpha* or classical satellite sequences located on specific chromosomes. The centromeric probes could be either pancentromeric probes or chromosome specific centromeric probes. Chromosome specific centromeric probes enable the detection of non-dysjunction and chromosome lagging thereby providing more information about the mechanism of action of the chemical. All of these techniques, although accurate, are more expensive and labour intensive.

4.125 NECROSIS AND APOPTOSIS

DNA damage, induction of necrosis and apoptosis are closely interrelated. Induction of necrosis can result in the release of degradable enzymes which can lead to partial digestion of DNA during early stages of necrosis. DNA damage by a genotoxic compound can lead to apoptosis. Inhibition of apoptosis may allow cells that have undergone DNA damage to proceed through the cell cycle and survive as mutated / micro-neucleated cells. Therefore in order to get a complete picture of a Genotoxic

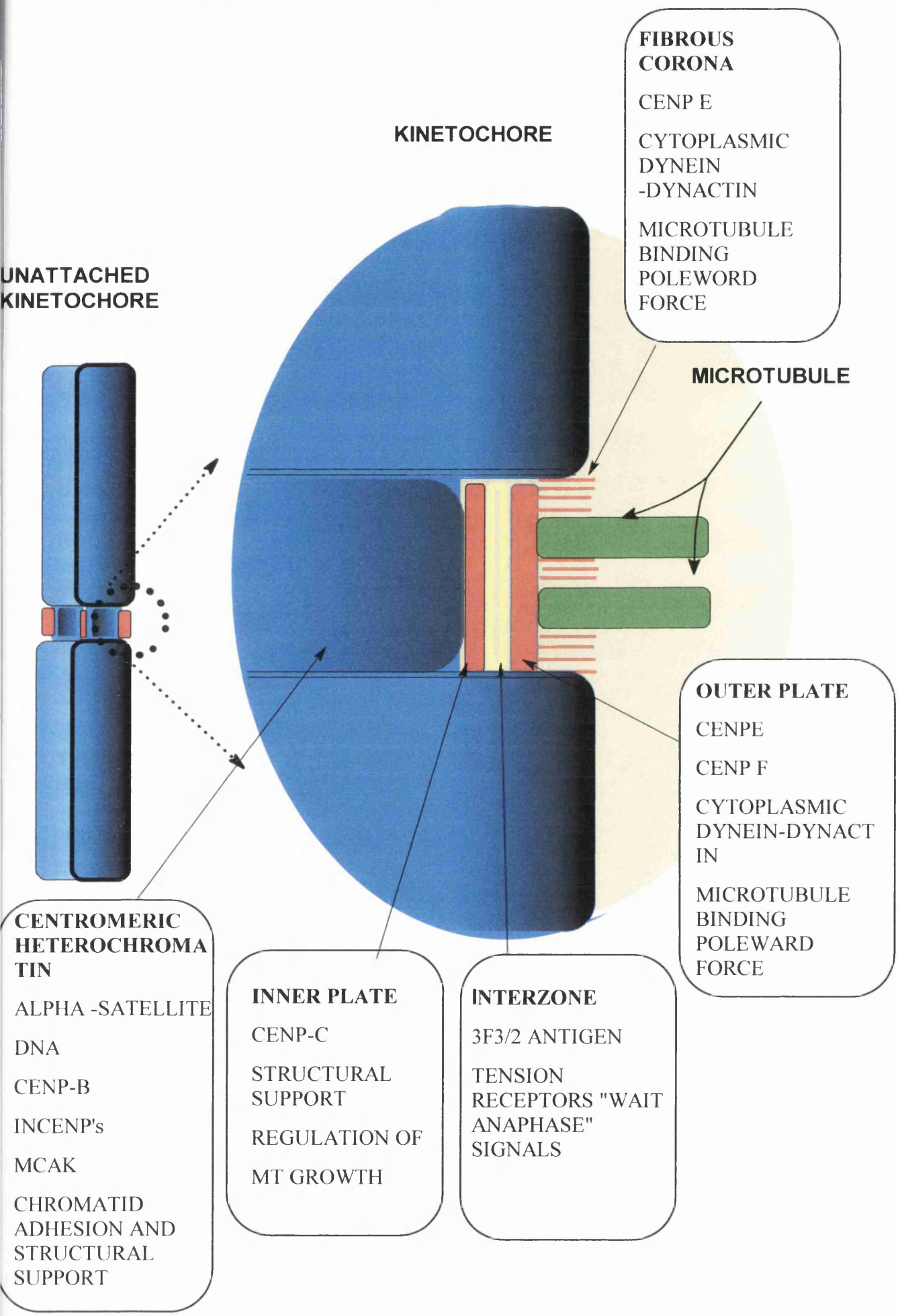
ability of an agent necrosis and apoptosis have to be measured concurrently. One of the unique advantages of CBMN assay is that it allows parallel estimation of mitotic delay apoptosis, necrosis and chromosomal aberration. Apoptotic cells can be detected as small cytoplasm condensed dark violet cells which show nueclei with different degrees of chromatin condensation and fragmentation depending on the stage of apoptosis. Necrosis is characterised by a loss of membrane integrity and release of toxic cytoplasmic enzymes into the surrounding tissue. Apoptosis, on the other hand, is characterised by preservation of the plasma membrane until late in the process without the leakage of intracellular complex.

4.13 BILE ACIDS

4.131 BILE ACIDS SYNTHESIS, STRUCTURE, FUNCTION AND 4.132 METABOLISM

The two principal bile acids formed in the liver are cholic acid and chenodeoxycholic acid. These are called the primary bile acids and are formed in the pericentral hepatocytes from cholesterol by a multi-enzyme process. In the synthesis of C24 bile acids the side chain of cholesterol undergoes oxidative cleavage resulting in the conversion of an iso-octane moiety into an iso-pentanoic acid moiety. One or two hydroxy groups are added to the nucleus (see figure 4.2 and table 4.3). The hydroxylation is always on one face of the molecule, and the final product invariably has a hydrophobic face and a hydrophilic face resulting in amphipathic molecule (Hofmann 1999). All bile acids are conjugated with either glycine or taurine resulting in glycocholic acid or taurocholic acid. Conjugation increases aqueous solubility at acidic pH, increases resistance to precipitation by Ca+, and renders bile acids impermeable to cell membranes.

Figure 4.1. Showing the putative location and possible function of reported kinetochore proteins. Modified from Rieder *et al* 1998



In the colon, bacteria convert (by the action of 7-dehydrogenase enzyme) cholic acid to deoxycholic acid and chenodeoxycholic acid to lithocholic acid (Ganong 1989; Hofmann 1999). These bile acids, namely deoxycholic acid and lithocholic acid are called secondary bile acids.

4.133 FUNCTIONS

The bile acids play an important role in the digestion and absorption of fat in the body. The bile acids reduce the surface tension and, in conjunction with phospholipids and monoglycerides, are responsible for the emulsification of fat preparatory to its digestion and absorption in the small intestine. The bile acids because of their amphipathic nature (i.e. they have both hydrophobic and hydrophilic domains) tend to form cylindrical disks called micelles, with hydrophilic surfaces facing out and the hydrophobic surfaces facing in. The micelles play an important role in keeping lipids in solution and transporting it to the intestinal brush border where they are absorbed (Ganong 1989).

Table 4.3: The Human Bile Acids (adapted from Ganong WF, 1997). The numbers refer to the position in the steroid ring

BILE ACIDS	Group at 3	Group at 7	Group at 12	% in human bile
Cholic acid	OH	OH	OH	50
Chenodeoxycholic acid	OH	OH	H	30
Deoxy cholic acid	OH	H	OH	15
Lithocholic acid	OH	H	H	5

4.134 TRANSPORT

90-95% the bile salts are absorbed from the small intestine. The remaining 5-10% of the bile salts that enter the colon are converted to the salts of deoxycholic acid and

lithocholic acid. Lithocholate is relatively insoluble and is mostly excreted in the stools. Deoxycholate on the other hand is absorbed. The absorbed bile salts are transported back to the liver and re-excreted in the bile (enterohepatic circulation). The total bile salt pool is approximately 3.5 g and the entire pool recycles about six to eight times a day (Ganong 1989).

4.14 BILE ACIDS AND CARCINOGENICITY

Cook and Kennaway had proposed the role of bile acids in carcinogenesis as early as 1932. They noticed that the bile acids had structural resemblance to carcinogenic polycyclic aromatic hydrocarbons. This was confirmed by the finding that subcutaneous injection of DCA in mice can lead to "malignant spindle celled tumours" (Cook *et al.*, 1940). Bile acids have long been implicated in the aetiology of colon cancer (Aries *et al.,* 1969; Wynder 1969; Hill *et al.,* 1971). Studies have shown that bile acids are either mutagenic or carcinogenic in experimental models. The bile acids have been implicated in carcinogenic process as mutagens or carcinogens. Several bile acids have been tested in Ames mutagenicity assay with negative results (Silverman *et al.,* 1977; McKillop *et al.,* 1983). Kulkarni *et al* showed tht lithocholic acid induces single strand breaks in Ll1210 cells (Kulkarni *et al.,* 1980). Watanabe *et al* showed that several bile acids were mutagenic to *Salmonella* using a fluctuation test (Watabe *et al.,* 1985). More recently, DCA and LCA have been shown to induce DNA damage in the alkaline comet assay. This genotoxic effect was significantly increased after treatment with endonuclease III, an enzyme that nicks DNA at sites of oxidized pyrimidines, and thus indicates the induction of oxidative base damage (Venturi *et al.,* 1997).

Recent studies using comet assay has shown that the bile acids induce DNA damage and apoptosis in cells in-vitro (Powolny *et al.,* 2001; Glinghammar *et al.,* 2002; Jolly *et al.,* 2004). Although there is growing evidence for DNA damaging ability of DCA there are reports that suggest that bile acids may not be genotoxic((Mori *et al.,* 1991; Venturi *et al.,* 1997; Fein *et al.,* 2000). Scates *et al* have shown that bile acids do not form DNA adducts. Studies have shown that bile acids do not damage DNA directly but require key cell based intermediates (Scates *et al.,* 1996; Glinghammar *et al.,* 2002). It has been shown DCA can cause damage by generating ROS (reactive oxygen speices) (Patel *et al.,* 1997; Fang *et al.,* 2004). The ROS inturn can cause oxidative DNA damage which could explain the mechanism of genotoxicity of DCA (Bernstein *et al.,* 2005).

4.141 MECHANISM OF CYTOTOXICITY

The exact mechanism of injury by bile acids is not known. There are two possible hypotheses.

- According to one hypothesis bile acid induced cell damage is probably due to their detergent property and solubilisation of mucosal lipid membranes. This theory is supported by studies in gastric mucosa where bile acid induced mucosal injury correlated with the release of phospholipids and cholesterol into the lumen. Experiment with rabbit mucosa has shown that bile acids cause disruption at a concentration which is much lower than the one required for phospholipid solublisation. Therefore this is a less likely mechanism of bile acid induced cell injury (Vaezi MF 2001; Bernstein *et al.,* 2005).

- The second and more favoured mechanism is that bile acids enter the mucosa due to their lipophilic nature causing intra-mucosal damage by disorganizing

membrane structures or interfering with cellular function (Vaezi MF 2000 Oct; Bernstein *et al.,* 2005). This model is supported by studies conducted by Batzri *et al* and Schweitzer *et al* who demonstrated that bile acids penetrated the mucosal barrier and were trapped inside the cells due to intracellular ionisation thus increasing the intracellular concentration of bile acids by several folds (Batzri *et al.,* 1991). Studies conducted by Schweitzer *et al* have correlated bile acid entry and mucosal accumulation with bile acid mediated mucosal damage (Schweitzer *et al.,* 1987).

4.142 MECHANISM OF CARCINOGENICITY (PROMOTER / GENOTOXIC ACTION)

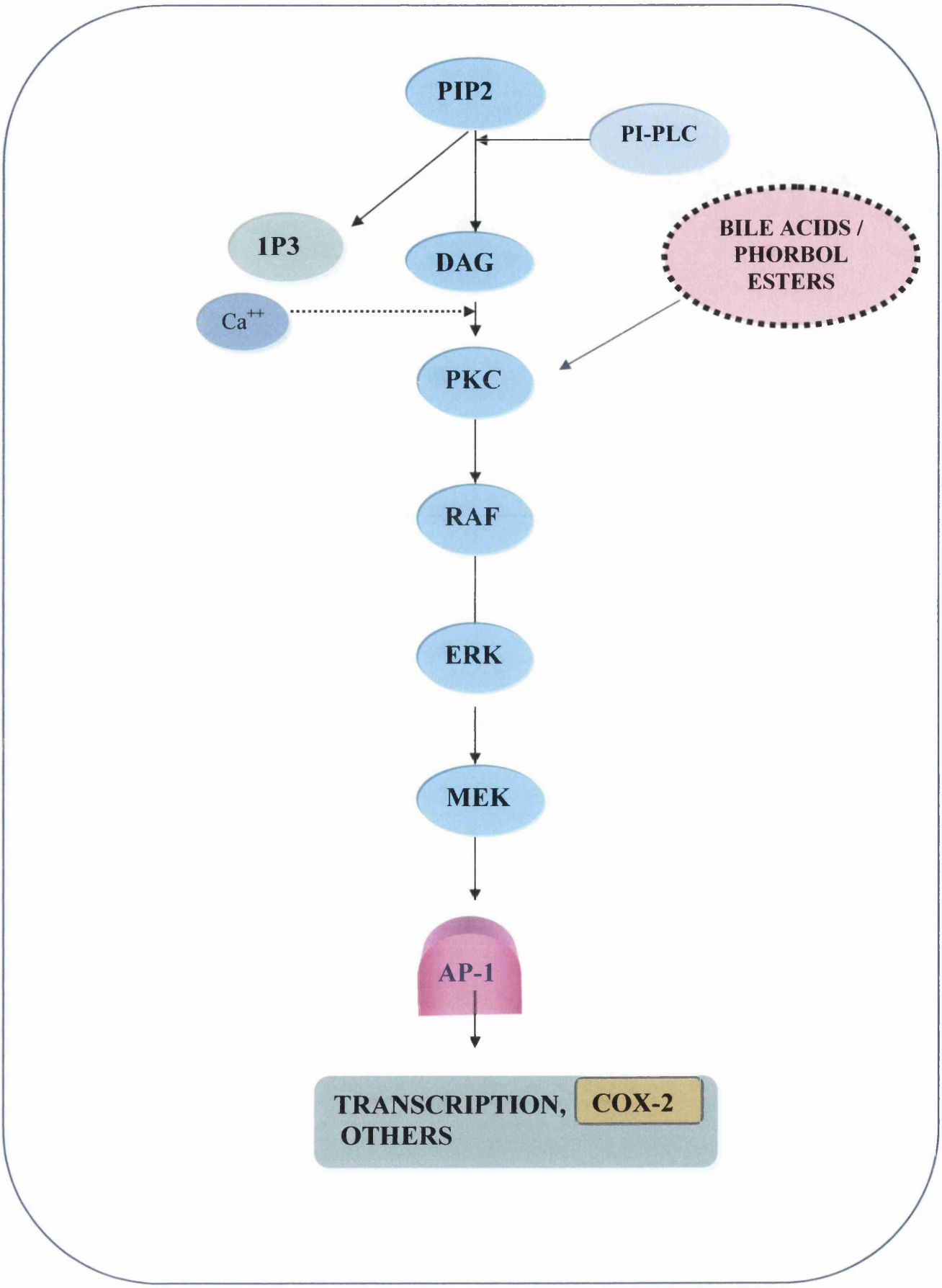
There has been conflicting opinion about the direct genotoxic effect of bile acids. Their exact role in the process of carcinogenesis is not clear. Epidemiological and animal studies have suggested that bile acids act as tumour promoters in the gastrointestinal tract (see section 4.182). The exact mechanism of their action is however not very clear. They probably act by affecting intracellular signalling and gene expression which leads to alteration in cell proliferation and cell death (Debruyne *et al.,* 2001; Qiao *et al.,* 2002; Bernstein *et al.,* 2005).

PROTEIN KINASE C AND AP-1 PATHWAY

Bile acids are known to cause increased cell proliferation and apoptosis in cell lines depending on the concentration of the bile acids. The mechanism by which they induce these changes is probably by the activation of PK-C (Huang *et al.,* 1992; Pongracz *et al.,* 1995; Hirano *et al.,* 1996; Rao *et al.,* 1997; LaRue *et al.,* 2000). Protein Kinase C comprises of a group of more than 11 phospholipid dependent ser/thr kinases. The PKC's are divided into three broad categories namely, conventional PKC's, novel PKC's and atypical PKC's depending on their need for

calcium and DAG (Diacyl glycerol) for activation. PKC are activated by a number of extra-cellular signals and play a critical role in many signal transducing pathways in the cell [Reviewed by (Koivunen *et al.,* 2005)]. They are involved in a variety of cell regulatory pathways, including mitogenesis, cell adhesion, apoptosis, angiogenesis, invasion and metastasis (Di Mari *et al.,* 2005). The classical PKC are activated by DAG and calcium in the presence of phosphatidyl serine. DAG and inositol 1-4-5-triphosphate (IP3) are generated from plasma membrane associated phosphatidylinosito l-4-5-diphosphate (PIP2) by the activation of phospholipase C (PLC). Ca ++ is released from an intracellular storage pool stimulated by IP3. Phorbol ester (plant derived diterpenes) mimics the action of DAG, but its action is sustained leading to long term activation of PKC (Musashi *et al.,* 2000). The bile acids probably stimulate PKC's in a phorbol ester like manner (see figure 4.3) by mimicking the action of DAG (de Kok *et al.,* 2000; Debruyne *et al.,* 2001). The bile acids may increase PKC activity by facilitating its association with phospholipids or by stimulating PI-PLC and thereby increasing the formation of DAG (Debruyne *et al.,* 2001). Bile acids also stimulate activator protein-1 (AP-1) which is probably mediated by stimulation of PKC and ERK (Extracellular signal Related Kinases) (Song *et al.,* 2005; Bernt *et al.,* 2006). Activator protein-1 (AP-1) is a collective term for dimeric transcription factors composed of Jun, Fos or ATF (Activating Transcription Factor) proteins. c-*fos* is required for the viability, proliferation and differentiation of cells, particularly bone and neuronal cells (Ameyar *et al.,* 2003; Eferl *et al.,* 2003). The jun components are essential for growth and proliferation. The AP-1 complex activated by bile acids consists of Jun D, Fra-1 and c-fos. Studies suggest that these proteins are involved in tumorigenesis (Eferl *et al.,* 2003). Hence this could be an important step in the tumorigenesis. COX-2 gene could be one of the important targets and mechanism of action of the bile acids (see figure 4.4). Increased expression of COX-2

Figure 4.3 Possible pathways involved in bile acid signalling [adapted and modified from (Debruyne *et al.*, 2001)]



is seen in a variety of cancers (colonic cancer, gastric cancer and oesophageal cancer). Although COX-2 is constitutively expressed in the normal oesophageal and duodenal mucosa, studies have shown that it is over expressed as an early event in the neoplastic transformation of BE (Shirvani *et al.,* 2000; Zhang *et al.,* 2001).

The induction of COX-2 by bile acids has been shown to be mediated via PKC dependent mechanism (Zhang *et al.,* 1998; Kaur *et al.,* 2002)). Li *et al* showed that bile acids could induce COX-2 in various oesophageal cell lines leading to an increased production of prostaglandin E 2. studies have shown that COX-2 inhibitors could act as chemopreventive agent in oesophageal cancer (Li *et al.,* 2000; Altorki 2004).

Cyclo-oxygenase is the key enzyme required for the conversion of arachidonic acid to prostaglandins. The COX pathway consists of two different iso-enzymes, COX-1 and COX-2. The recently identified COX-3 is a splice variant of the COX-1 gene the exact function of this in humans remains to be elucidated (Tuynman *et al.,* 2004). COX-1 is expressed constitutively in most tissues and is responsible for the house keeping functions. COX-2 on the other hand is usually not detectable in most of the normal tissues but is induced by oncogenes, growth factors, carcinogens and tumour promoters. Both COX-1 and COX-2 are similar with only minor differences in their catalytic activity. COX-2 activation leads to enhanced synthesis of prostaglandins. The prostaglandins can promote angiogenesis, inhibit immune surveillance and increase cell proliferation. COX-2 over expression also inhibits apoptosis and increases the invasiveness of the malignant cells. The other mechanism by which COX-2 may function is lowering the level of arachidonic acid. High concentration of arachidonic acid can promote apoptosis. COX-2 catalysis pathway may simply

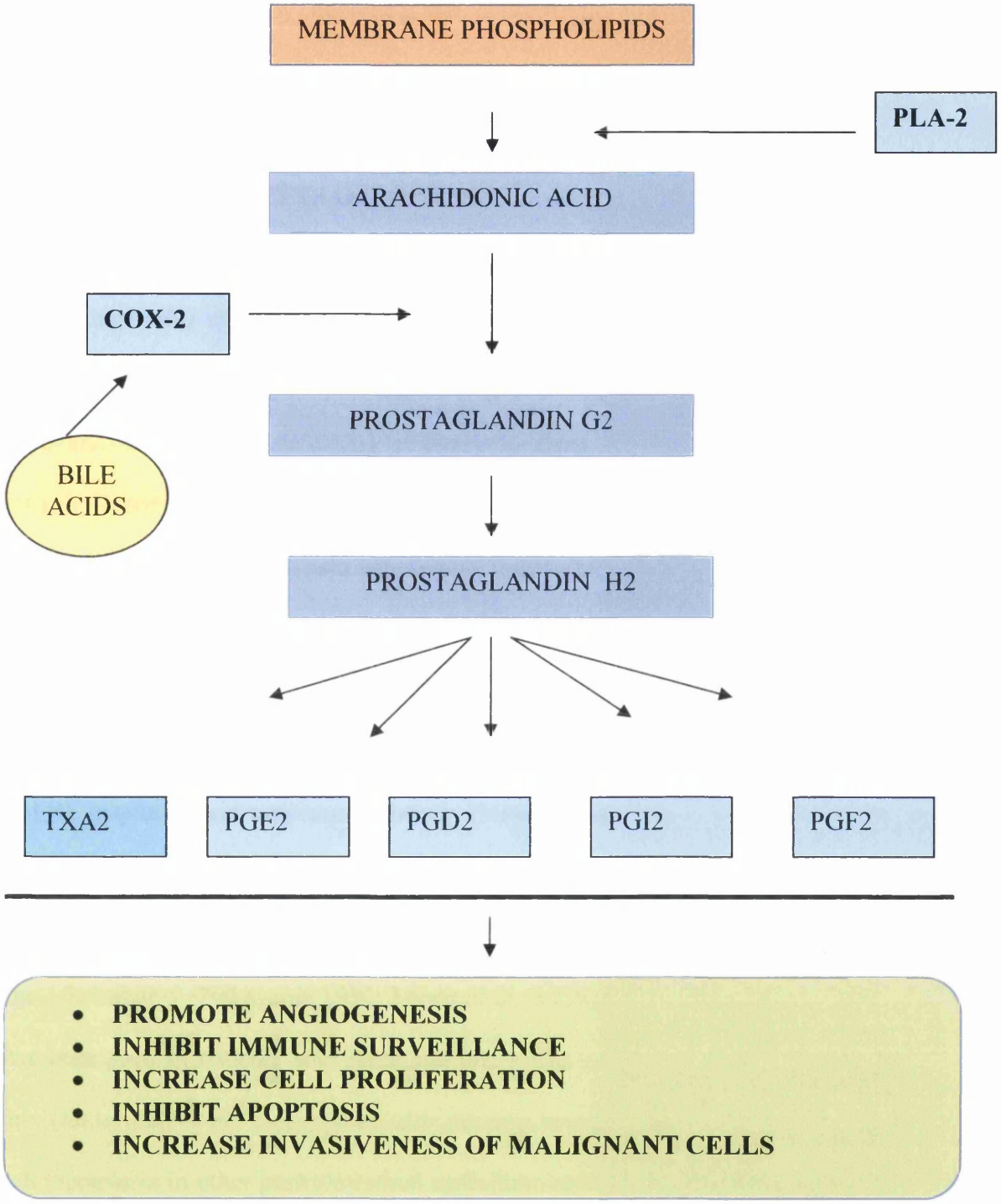


FIGURE 4.4: Mechanism of action of COX-2. Arachidonic acid is released from membrane phospholipids by phospholipase-A2 (PLA2) and is metabolized by cyclooxygenase (COX) to prostaglandin H2 (PGH2) in two steps. PGH2 is converted to a variety of eicosanoids by specific isomerases (Abbreviation: TXA2, thromboxaneA2). Adapted from (Subbaramaiah *et al.*, 2003)

deplete the apoptotic signal by depleting the concentration of arachidonic acid (Simmons *et al.,* 2004).

4.17 BILE ACIDS IN BARRETTS OESOPHAGUS

The exact aetiology of BE is still unknown. However it is clear that BE is associated with longstanding reflux of acid, bile and pancreatic secretions compared to normal patients. The exact role of individual constituents of the refluxate in the development of BE is unknown. There is increasing evidence to show that bile acids play an important role in the pathogenesis of BE and its progression to adenocarcinoma. If acid reflux alone induces BE then acid suppressive therapy should potentially reverse BE. Studies have shown that 25-35% of the patients on recommended dose of PPI's develop recurrent or progressive disease (Sontag 1992; Champion *et al.,* 1994; Jankowski *et al.,* 2000). None of the studies so far have shown complete regression or reversal of BE inspite of acid suppression therapy (Neumann *et al.,* 1995).

Oesophagitis has been seen in achlorhydric patients with pernicious anaemia (Palmer 1960; Orlando 1973). It has also been documented in patients with gastrectomy and oesophago-jejunostomy (Nakayama 1956; Meyer *et al.,* 1979). Neoplastic progression of BE has been seen in patients who have had bile reflux without any pathological acid reflux (Jankowski *et al.,* 1993). Bile acids are also involved in the promotion of goblet cell metaplasia in other gastrointestinal epithelium such as stomach duodenum, intestine and bile ducts. All of these factors suggest that bile acids alone could be highly important in the pathogenesis of BE and its complications.

4.15 DGER

Duodenogastric reflux was first observed and assessed by Beaumont in 1833. In the last few decades DGER has been associated with a number of pathologic conditions of the foregut such as gastric ulcer, gastritis gastric cancer and dyspepsia (Ritchie 1980; Schumpelick 1983; Du Plessis DJ 1962). Recent experimental and clinical evidence indicates that DGER plays a significant role in the development of Barrett's oesophagus and adenocarcinoma of the distal oesophagus (Johnson 1986; Ireland 1996; Pera 1998). The evidence for association of GERD and Barrett's oesophagus is over-whelming. However, the role of individual constituents of the refluxate in the development of BE and its complications (ulcer, strictures, dysplasia or adenocarcinoma) is not known. DGER is retrograde reflux of duodenal contents (bile and pancreatic fluid) into the stomach with subsequent reflux into the oesophagus. DGER occurs in normal individuals, particularly postprandially (Muller-Lissner 1983; Mackie 1986; King 1987) and at night (Gotley 1991; Nehra *et al.,* 1999). Terms such as 'Bile reflux' and 'alkaline reflux', which have been used in the past, are no longer appropriate. The term bile reflux is in-appropriate because duodenal contents contain more then just bile. The term alkaline reflux is a misnomer because recent studies have shown that DGER can occur at neutral as well as acidic pH (Nehra *et al.,* 1999). Further-more rise in pH does not predict the presence of bile, as oesophageal pH > 7 could be due to presence of saliva, secretions from the sub mucosal oesophageal glands, food or oral infection (Singh 1993). Studies have also shown that 70-91% of the DGER episodes occur in an acidic environment (Johnson 1997).

4.151 MEASUREMENT OF DGER

Extrapolating the results regarding the injurious effect of DGER from the animal data into humans has been hampered for a long time due to lack of "gold standards" for

detecting DGER in humans. A number of different methods have been described to assess DGER (see table 4.4). Some of the earlier methods used for measuring DGER such as endoscopy, aspiration studies (both gastric and oesophageal), scintigraphy and ambulatory pH monitoring have technical difficulties and are imprecise.

Endoscopic observation of bile pool in humans is a poor marker of bile induced oesophageal disease (Katz 2000; Vaezi 2001). Aspiration of gastric contents for measurement of bile acid and trypsin concentration, although helpful, represents only a short-term assessment of reflux of duodenal contents into the stomach occurring in a 24-hour period (Katz 2000; Richter 2001; Vaezi 2001). Scintigraphic evaluation for DGER is stationary and results in radioactive exposure to patients, and has conflicting results (Stein *et al.,* 1999; Katz 2000). Using pH > 7 ("alkaline reflux") as a marker for esophageal exposure to duodenal contents (DGER) has recently been shown to be an inaccurate means of detecting DGER for the reasons already discussed above.

There has been a renewed interest in DGER and role of bile in BE with the advent of the Bilitec 2000. Bilitec is a new fibreoptic spectrophotometric system which measures DGER independent of the pH. It utilizes the optical property of bilirubin the most common bile pigment. Studies indicate very good correlation between Bilitec reading and bile acid concentration as measured by duodenogastric aspiration studies (Vaezi 1995). However Bilitec 2000 tends to under-estimate bile reflux by almost 30% in an acidic medium i.e. at pH < 3.5. Furthermore the readings can be modified by the presence of some dietary substances (Vaezi 1995; Barrett *et al.,* 2000). Despite its limitations Bilitec 2000 is an invaluable device in the assessment of DGER.

4.152 ANIMAL STUDIES

Early studies by Cross and Wangensteen suggested a role for bile acids in oesophageal mucosal damage. Moffat and Berkas used a dog model with biliary diversion and a jejunal conduit anastomosing directly to the oesophagus to show that

canine bile was capable of producing various degrees of erosive oesophagitis, thereby confirming the earlier studies by Cross and Wangensteen (Vaezi *et al.,* 2000).

Shafie-Shirazi *et al* showed that bile acid combined with HCl was more damaging than HCl or bile acid alone (Safie-Shirazi 1975). More recent studies show that oesophageal mucosal damage by bile acids is dependent on the conjugation state of the bile acids and pH of the refluxate. Harmon *et al* showed that conjugated bile acids were more injurious to oesophageal mucosa at acidic pH, whereas un-conjugated bile acids were more harmful at pH 5-8 (Harmon *et al.,* 1981).

TABLE 4.4: SHOWING THE VARIOUS METHODS AVAILABLE FOR THE ASESSMENT OF DGER WITH THEIR RELATIVE ADVANTAGES AND DIS- ADVANTAGES

METHOD	ADVANTAGES	DISADVANTAGES
Endoscopy	Easy visualization	poor sensitivity/specificity/positive predictive value Requires sedation High cost
Aspiration Studies	Easy visualization of bile No sedation required Low cost	Short duration of study Requires familiarity with enzymatic assay for bile acid
Scintigraphy	Noninvasive	Semiquantitative at best Radiation exposure High cost
pH monitoring	Easy to perform Relatively noninvasive Prolonged monitoring	pH >7 not a marker for DGER Nonspecific for DGER
Bilirubin Monitoring (Bilitec 200)	easy to perform relatively noninvasive prolonged monitoring good correlation with gastric bile acid concentrations.	Current design under estimates DGER by about 30% in acidic medium(pH 3.50) Requires modified diet

The pH dependent damage caused by conjugated and un-conjugated bile acids was further confirmed by Kivilaasko *et al* using rabbit oesophageal perfusion studies (Kivilaakso 1980).

Lillemoe *et al* compared the injurious effects of various duodenal components on rabbit oesophageal mucosa at pH 2. At this acidic pH trypsin had no effect on the net flux of ions across the oesophageal mucosa, as it is inactive at pH <4. At neutral pH taurocholate showed no effect, but in an acidic medium there was mucosal disruption. There was also evidence of synergism between pepsin and HCl as well as between HCl and conjugated bile acids in causing oesophageal mucosal damage(Lillemoe 1982). Table 4.5 summarizes some of the animal studies conducted so far. A number of experimental animal models have been used to study the effects of DGER. Studies in rat have shown that reflux of duodenal contents, produced by means of oesophagojejunal anastomosis, exerts a co- carcinogenic effect with 2,6 –dimethyl nitrosomorphine (2,6-DMNM) (Pera M 1989; Attwood *et al.,* 1992). Goldstein et al surgically induced DGER by oesophagoduodenostomy in Sprague Dawley. They found that DGER induced changes similar to BE and adenocarcinoma in the rats (Goldstein *et al.,* 1997).

Melo *et al* studied the effect of gastro-oesophageal reflux and DGER in female wistar rats by performing cardioplasty and oesophagoduodenostomy. They found that DGER in isolation behaved as an oesophageal carcinogen by inducing oesophagitis, BE and oesophageal adenocarcinoma in the rats. Oesophageal carcinogenesis induced by DGER was potentiated by DEN (diethyl nitrosamine) but DEN in isolation failed to induce carcinoma (Melo 1999).

Fein *et al* studied the effects of artificially induced DGER in rats. In their study DGER induced cancers in the rat oesophagus, but the duodenal fluid from the rats

failed to show any genotoxicity in the mouse lymphoma micronucleus assay. All their samples showed cytotoxic effect but none of them had any genotoxic effect (Fein *et al.,* 2000). They concluded that EAC in the experimental model was due to chronic inflammation rather than due to presence of any specific carcinogen.

Table 4.5 EFFECTS OF COMPONENTS OF DUODENAL REFLUXATE AND SURGICAL PROCEDURE IN ANIMAL MODELS

Authors	Animal/ Procedure	Component Evaluated	Damaging effects/ Outcome
Safaie-Shirazi et al. 1975	Rabbit	Bile, HCl	Bile acid plus HCl is more damaging than HCl or bile acid alone.
Kivilaakso et al. 1981	Rabbit	Conjugated or un unconjugated bile acids, pepsin, lysolecithin	Conjugated bile acids and pepsin at pH 3.5; uncon-jugated bile acids at pH 7; lysole-cithin and trypsin at pH 7.
Harmon et al. 1981	Rabbit	Conjugated bile acids, unconjugated bile acids or HCl	HCl alone damages only at pH 1; conjugated bile acids at pH 2; unconjugated bile acids at pH 7.
Lillemoe et al. 1982	Rabbit	Pepsin, conjugated bile acid, trypsin, HCl	At pH 2, pepsin > conjugated bile acid > trypsin > HCl.
Salo and Kivilaakso 1984	Rabbit	Trypsin, unconjugated bile acid	At pH 2, trypsin plus unconjugated bile acid >trypsin > conjugated bile acid.
Pera et al , 1989	Sprague Dawley rats; oesophago-jejunostomy	DGER + DMNM(a nitrosamine)	Induced EAC in 24% of the rats
Miwa et al ,1996	Wistar rats; esophago-jejunostomy	DGER	92-100% BE, 75% EAC
Goldstein et al, 1997	Sprague Dawley rats Oesophago-duodenostomy	DGER	BE and EAC
Fein et al, 2000	Sprague Dawley rats Oesophago-duodenostomy	DGER	BE and EAC

More recently similar experiments performed by Pera *et al* showed that duodenal reflux alone in rats can induce metaplastic changes and oesophageal cancers (Pera M 2000). These tumours could range from squamous carcinoma to adenosquamous carcinoma to adenocarcinoma. Based on their experiments proposed that chronic duodenal reflux induces the development of metaplastic cells, which in turn can progress to adenocarcinoma. **CDCA** an unconjugated bile acid was shown to promote duodenal tumours in an animal model of familial adenomatous polyposis (Mahmoud *et al.*, 1999).

4.153 HUMAN STUDIES

As already described various methods have been used to detect bile reflux in the oesophagus. With the use of Bilitec 2000 it has been possible to monitor bile reflux in an ambulatory setting, independent of the pH over a 24 hr period. Using Bilitec 2000 Kauer *et al* found that patients with Barrett's had more acid and bile reflux than normal patients (Kauer *et al.,* 1995). Champion *et al* observed a graded increase in bile reflux and acid reflux from controls to patients with reflux oesophagitis with the highest values observed in patients with Barrett's oesophagus (Champion *et al.,* 1994).

Subsequent studies by Vaezi *et al* and Richter *et al* have supported the graded increase in bile reflux. Not only did the patients with Barrett's have increasing and acid reflux, but also had episodes of acid reflux which paralleled episodes of bile reflux (Vaezi *et al.,* 1996; Richter 2001). Bilitec 2000 is qualitative rather than a quantitative test for bile acids. Bilitec cannot distinguish between the different bile acids nor can it distinguish between conjugated and unconjugated bile acids.

Gotley *et al* tried to measure the concentration of bile acids in patients with DGER. They performed continuous aspiration studies. They found that the refluxate contained

predominantly conjugated bile acids and the bile reflux was particularly severe during the nocturnal periods. More than 24% of their patients with reflux oesophagitis had a total bile reflux of >200μmol/L during the nocturnal period (Gotley *et al.,* 1988). Iftikhar *et al* measured the concentration of the various bile acids in patients with BE (Iftikhar *et al.,* 1993). Their studies showed that patients with BE had a higher bile reflux and the most predominant bile acids were glycocholic and taurocholic acids. In patients with BE, the bile acids were found in a concentration ranging from 0-14000μmol/L.

More recently Nehra *et al* studied the concentration of various bile acids in patients with reflux oesophagitis and in patients with Barrett's oesophagus by continuous aspiration of the bile acids over a period of 15 hours. They found that bile acids refluxed concurrently with acid in most instances. Patients with BE and reflux oesophagitis had increasing bile reflux compared to normal patients. They also found that in 20% of the patients with BE bile reflux occurred independently without any acid reflux. They found that patients with BE had an average bile reflux of 180μmol/l and a maximum of about 800- 1000μmol /l. More than 50% of their patients with BE had a bile reflux exceeding 200μmol/l (Nehra *et al.,* 1999). The predominant bile acids detected in the patient groups consisted of primary bile acids, cholic acid, taurocholic acid and glycocholic acid. A significant proportion of patients with extensive mucosal injury had increased taurodeoxycholic acid, cholic acid and deoxycholic acid (Nehra *et al.,* 1999). Kauer *et al* showed that majority of the duodenal reflux occurs at a **pH range of 4 to 7** (Kauer *et al.,* 1997).

A number of in vitro experiments have been conducted to study the effects of bile acids on Barrett's epithelium. Bachir *et al* studied the effect of conjugated bile acids on oesophageal tissue in organ culture. They used taurine and glycine conjugated bile salts in concentrations ranging from 1mmol/L up to 3mmol/L. They found that

concentrations of bile acids over 3mmol had a generalized cytotoxic effect where as concentrations of 1-2mmol had a milder cytotoxic effect seen in the form of progressive and consecutive shedding of the superficial layers of the epithelium (Bachir *et al.,* 1982). As already described Shirvani *et al* have showed that pulses of acid and bile increased the expression of COX-2 in BE explants (Shirvani *et al.,* 2000). More recently Kaur *et al* showed that exposure of BE explants to pulses of bile salts increases the proliferation, whereas exposure to a combination of bile salts and acid together inhibits proliferation (Kaur *et al.,* 2000). They used glycine and taurine conjugated cholic and chenodeoxycholic acid in the final concentration of 1mmol/L. The cell proliferation was associated with increased level of PKC (Kaur *et al.,* 2000). Dixon *et al* studied biopsies (both gastric and oesophageal) from patient with BE and reflux oesophagitis for reflux gastritis score (RGS) and bile reflux index (BRI). Based on these parameters they showed that patients with BE had more evidence of bile gastritis than patients with uncomplicated GORD suggesting that bile could be an important factor in the development of BE and dysplasia (Dixon *et al.,* 2001).

AIMS:

The aim of this chapter was to evaluate the cytotoxic and genotoxic effects of various bile acids commonly seen in patients with BE. The effects of these bile acids were evaluated in concentration which was physiologically relevant.

4.2 MATERIALS AND METHODS

4.21 CULTURE OF OE33 CELLS

The OE33 cells were cultured in monolayer in 25 cm^2 tissue culture flasks (Nunclon). The techniques of culture, storage and thawing of these cells have been described in detail in Chapter 3 section 3.31.

4.22 IN VITRO MICRONUCLEUS ASSAY WITH OE33 CELLS

Flasks with confluent growth were selected (50 cm^2 or 75 cm^2) for the initiation of fresh culture. The culture flasks were split with trypsin EDTA as described in the earlier section. The cells were seeded in a concentration of 1-2 x 10^5 cells /ml into several 25 cm^2 flasks to make the final quantity of the culture medium to 10mls in each flask. Flasks were gassed and returned to the incubator at 37°C. Twenty four hours after the initiation of the culture chemical treatments were added (1%v/v, 100ml /10ml culture). Cytochalasin-B was added to the culture flasks in a final concentration of 4μg/ml (ie.40Mg/10ml) 44 hours after the initiation of culture. The cells were harvested at 72 hrs from the time of initiation of cultures (figure 4.5).

The toxic culture medium from each flask was discarded into appropriately labelled universals. The cells were then split with trypsin EDTA as described previously. The trypsin washes were discarded into the respective labelled universals. After the final trypsin wash all the detached cells were transferred into the respective universals and centrifuged at 1500RPM for about 8 minutes. The supernatant was discarded and the pellets were re-suspended in RPMI 1640 with 5% foetal calf serum.

4.221 SLIDE PREPARATION

The slides were prepared in a similar manner for all the experiments. The detached cells were washed and re-suspended in RPMI 1640 and 5% FCS in a concentration of about 1-2 x 10^5 cells/ml. Aliquots of 100-200μl were centrifuged in the cytospin (Shandon) for 8 minutes at 1200RPM. The quality of the slides was checked under the simple microscope. The slides were then air dried for a brief period and then fixed with 90% methanol at -20°C for 10 minutes. 6-8 slides were prepared for each replicate. After fixing the cells were air dried. Slides for Giemsa staining were stored at room temperature while slides for kinetochore labelling were stored at -20°C.

4.222 STAINING OF THE SLIDES (GIEMSA STAINING)

The slides for Giemsa staining were fixed with methanol at -20°C, dried and stored at room temperature. These slides were first immersed in xylene (Fisher Scientific Ltd UK) for about ten seconds and then allowed to dry. These slides were then stained with 10% Giemsa (Gurr BDH) in phosphate buffer at pH 6.8. The phosphate buffer was prepared by dissolving a phosphate tablet (BDH) in one litre of deionised water. The slides were immersed in the 20% Giemsa solution for eight minutes. The slides were then washed a few times with the phosphate buffer and water to wash off the excess of the stain. Once dry, the slides were mounted with a few drops of DPX mounting medium (BDH) and covered with glass cover slips. They were then left for a few hours to dry. The slides were then visualised and scored under an Olympus BH2 (Dixon *et al.,* 2001)

4.223 KINETOCHORE LABELLING

Slides were prepared and stored at –20 °C as described in the previous section. The kinetochores were detected by immunofluorescence method. This method enables the detection of chromosome loss. The protocol employed was similar to the one described by Ellard *et al* in 1991 with minor modifications. The slides were removed from -20°C and re-hydrated in PBS at room temperature in a coplin jar for about a minute. The slides were then placed in 0.5µg/ml primulin (Sigma) in deiodinised water for about 30 seconds. The anti-kinetochore antibody (Antibodies Incorporated) was prepared by diluting with PBS in a 1:1 ratio. 50µl of the antikinetochore antibody was added to each slide and covered with half size plastic cover slip. The slides were then incubated at 37°C for 45 minutes. After incubation for 45 minutes three washes were made for 3 min each in bovine albumin serum solution (1% solution prepared in

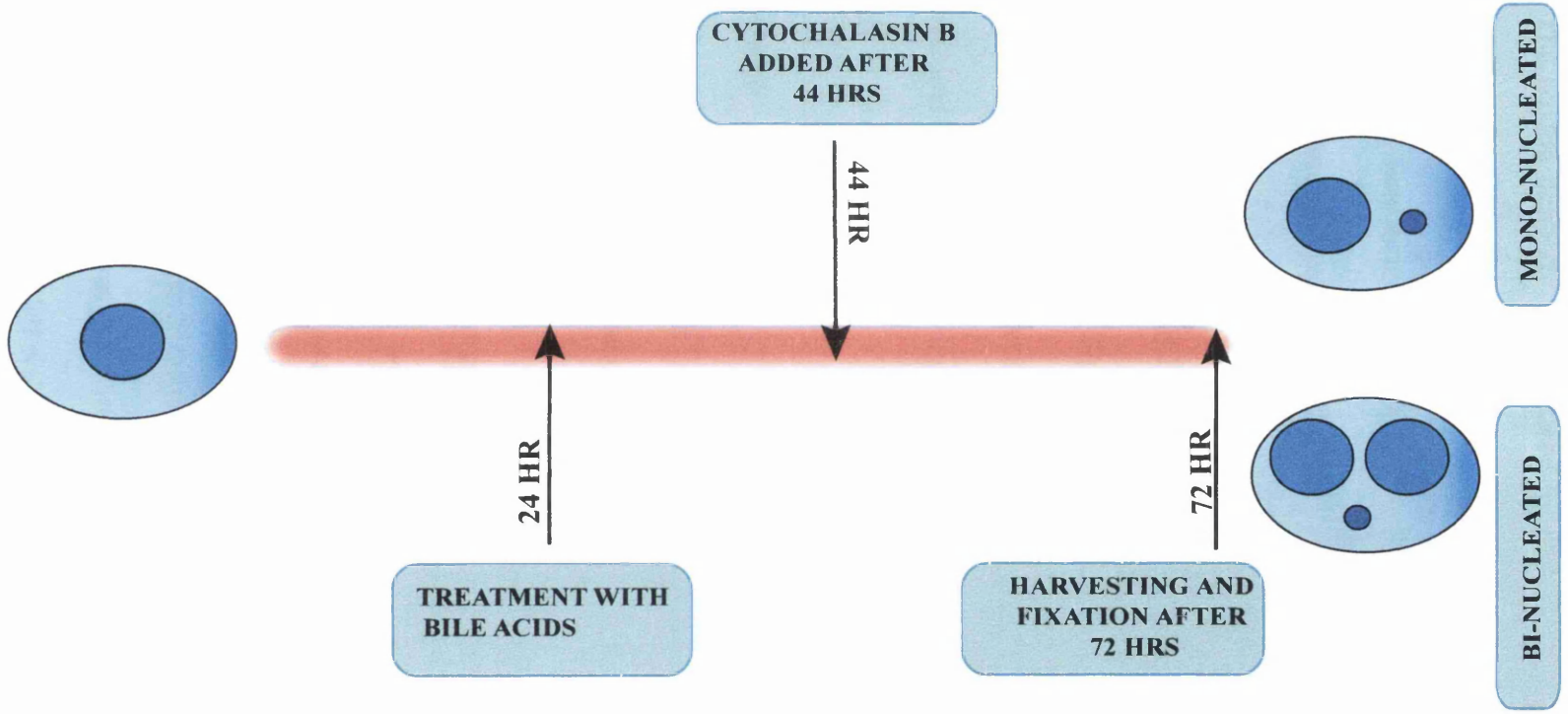


FIGURE 4.5. SHOWING THE TREATMENT AND HARVEST TIMINGS FOR THE OE33 MN ASSAY WITH BILE ACIDS

PBS) + PBS followed by three washes of three minute each in PBS at room temperature. The second antibody was prepared by diluting 1µl of Antihuman IgG Fluorescein Isothiocyanate (FITC) conjugate (Sigma) in 99µl of PBS. The slides were drained briefly, the dot was located and 50µl of the second antibody was added to each slide. The slides were covered with half coverslips and incubated at 37°C for 45 minutes. After that three washes were made for 3 min each in bovine albumin serum solution followed by three washes fort 3 minutes each in PBS. The slides were air dried and stored in the dark at 4°C. 20µl of DAPI was added to each slide before scoring. DAPI was prepared as follows:- 1µl DAPI + 9µl d H_2O + 990µl mounting medium (Vectashield Vector). The slides were left in the dark for 10 minutes before they were ready to be scored.

4.23 CHEMICALS

4.231 MITOMYCIN C

Mitomycin C is a genotoxic antineoplastic agent derived from *streptomyces caespitosus.*

It is used as a primary chemotherapy agent for anal, lung and superficial bladder cancers and as a secondary agent in breast, colon, gastric and pancreatic cancers (Verweij *et al.,* 1990). MMC is a potent mutagen and has been shown to be a predominantly clastogenic agent (Rudd *et al.,* 1991; Nesti *et al.,* 2000) MMC was used a **positive control** in all the experiments. MMC was used in the concentration of about 10-100µg/l. Mitomycin C was purchased from Sigma. Stock solutions of MMC were prepared by dissolving in distilled water.

4.232 BILE ACIDS

Six different bile acids were used in this study namely:

- Cholic Acid Sodium Hydrate (Sigma Aldrich Co Ltd. Dorset, UK)

  Molecular formula: C24H39NNaO5

  Molecular weight: 430.6

- Deoxycholic Acid (Sigma Aldrich Co Ltd. Dorset, UK)

  Molecular formula: C24H39NaO4

  Molecular weight: 432.6

- Glycocholic Acid sodium (Sigma Aldrich Co Ltd. Dorset, UK)

  Molecular formula: C26H42NNaO6

  Molecular weight: 487.6

- Taurocholic Acid, Sodium Salt Hydrate (Fisher Scientific UK)

  Molecular formula: C26H44NNaO7S

  Molecular weight: 537.7

- Glycodeoxycholic Acid Sodium (Sigma Aldrich Co Ltd. Dorset, UK)

  Molecular formula: C26H42NNaO5

  Molecular weight: 471.6

- Taurodeoxycholic Acid (Sigma Aldrich Co Ltd. Dorset, UK)

  Molecular formula: C26H44O6SNa

  Molecular weight: 521.7

Stock solutions of all the bile acids were prepared by dissolving in distilled water. Dilutions were made as needed to add no more than 100 µl to each flask containing 10ml of the medium. Untreated cultures were used as **negative controls**. All compounds were tested in two different experiments.

The experiments were conducted in two different culture conditions, at pH 7.3 (physiological) and at pH 5.5 (acidic). To obtain the acidic culture medium, RPMI 1640 was titrated with filtered 1M HCL to achieve a pH of 5.5 (measured with pH strips). A pH below 5 was found to be deleterious to the cells. The above bile acids

were used in our study as they are most commonly encountered in patients with reflux oesophagitis and BE (Gillen *et al.,* 1988; Iftikhar *et al.,* 1993; Nehra *et al.,* 1999; Stein *et al.,* 1999). The bile acid concentration 50μmol – 1m mol was selected as it represented the near physiological concentration of bile acids seen in patients with BE. All of the bile acids remained in solution and the solubility was not affected by the pH of the medium. Distilled water was used as a vehicle for dissolving the bile acids and Mitomycin C.

4.24 SCORING OF SLIDES

4.241 SCORING OF GIEMSA SLIDES FOR MICRONUCLEI

All the slides including the positive and negative controls were independently coded before scoring. 500 bi-nuecleated cells were counted per dose for each experiment. Two separate experiments were conducted for each dose (1000 cells per dose). This was followed for all experiments with bile acids (except GDCA) at neutral pH. For experiments with GDCA and all the other bile acids at acidic pH microneucli were scorred in a total of 1000 bi-nuecleate cells per culture (2000 cells per dose). The slides were scored in an Olympus BH2 microscope. Tri-nucleate, tetra-nucleate and multinucleate cells were also counted at the same time. Criteria described by M Fenech (2000) were used to identify binucleate cells and micronuclei.

4.242 CRITERIA FOR SELECTING BINUCLEATED CELLS

The bi-nucleated cells in the cytokinesis-blocked assay were scored based on the following criteria:

- The two nuclei in a bi-nucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary;
- The two nuclei in a bi-nucleated cell should be approximately equal in size, staining pattern and staining intensity;

- The two nuclei within a BN cell may be attached by a fine nucleoplasmic bridge which is no wider than 1/4th of the largest nuclear diameter.
- The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable.
- The cytoplasmic boundary or membrane of a bi-nucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

4.243 CRITERIA FOR SCORING MICRONUCLEI (MN)

MN are morphologically identical to but smaller than nuclei (figure 4.6). They also have the following characteristics:

- The diameter of MN should be no greater than a third of that of the main nuclei.
- MN are non-refractile and they can therefore be readily distinguished from artefact such as staining particles;
- MN are not linked or connected to the main nuclei ;
- MN may touch but not overlap the main nuclei and the micro-nuclear boundary should be distinguishable from the nuclear boundary;
- MN usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

4.244 SCORING OF APOPTOTIC AND NECROTIC CELLS

Apoptosis and necrosis were scored based on the criteria described by M Fenech *et al* (1999). Five hundred cells were counted and scored as either mono-nucleate, bi-nucleate, tri-nucleate, tetra-nucleate, apoptotic or necrotic and the ratio for these types of cells were calculated. Apoptotic and necrotic cells were identified based on the following features (figure 4.7).

Cells with chromatin condensation and intact cytoplasmic and nuclear boundaries ("early" apoptotic cells) or cells exhibiting nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane ("late" apoptotic cells) were counted as apoptotic cells.

Cells exhibiting a pale cytoplasm with numerous vacuoles (mainly in the cytoplasm and some in the nucleus) and damaged cytoplasmic membrane with a fairly intact nucleus ("early" necrotic cells) or cells exhibiting loss of cytoplasm and damaged/irregular nuclear membrane with only a partially intact nuclear structure ("late necrotic cells) were scored as necrotic cells.

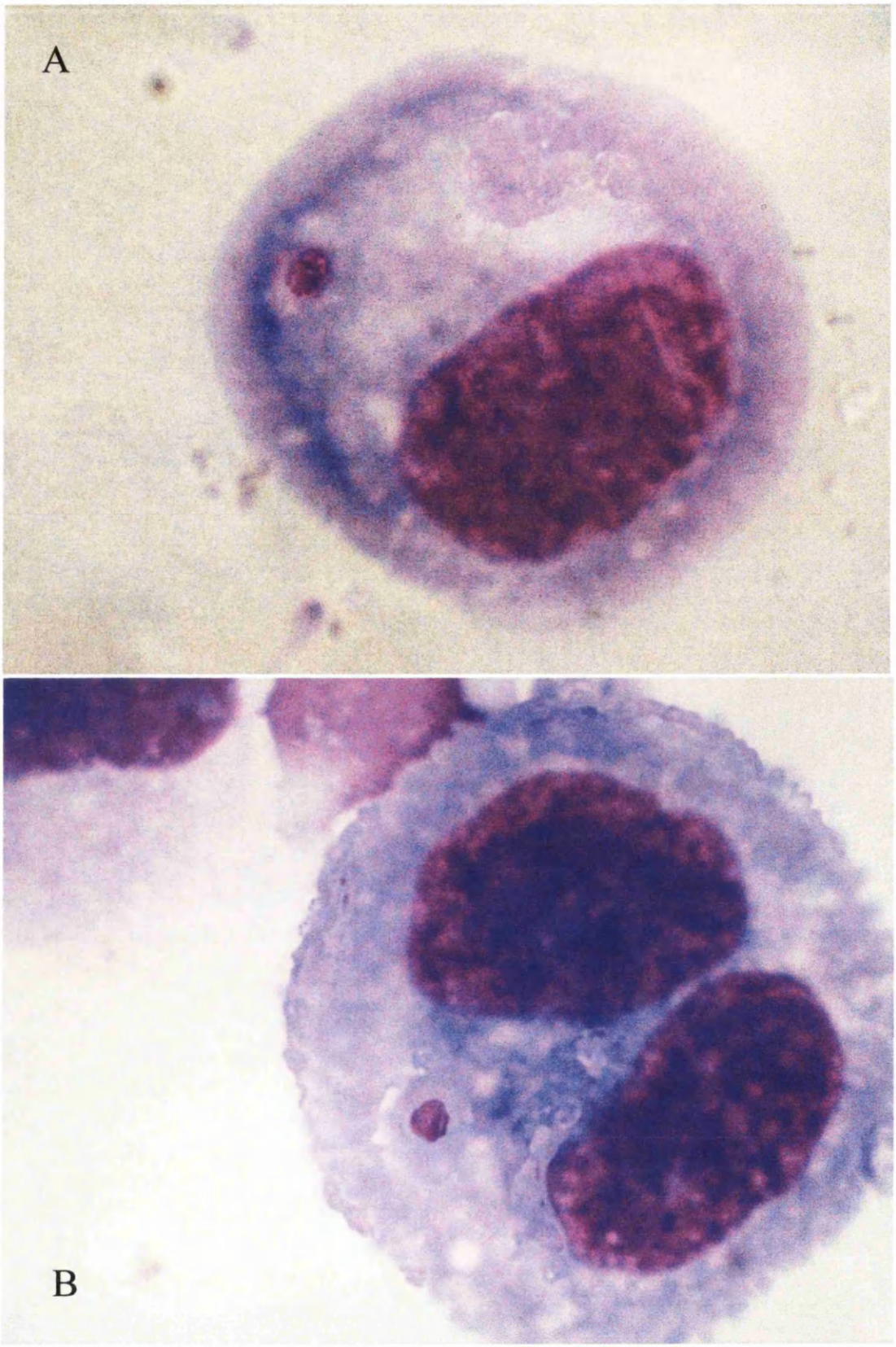
4.245 SCORING OF KINETOCHORE LABELLED SLIDES

Slides were labelled for kinetochore as described in the previous section. The slides were examined under fluorescence microscope using the filter for blue spectra in order to locate bi-nucleated cells containing MN. Upon location of an MN, switching filters to either the triple band pass filter or the single band filter for green spectra allowed the presence or absence of the green kinetochore signal to be detected. Scoring of kinetochore in a micronucleus was restricted to those cells which had good kinetochore signals (>20 in each nucleus) in their nuclei (figure 4.8). A minimum of 100 micronuclei were counted for either presence or absence of signals.

4.25 STATISTICAL EVALUATION OF THE DATA

The statistical difference between controls and each treated sample were identified with the one tailed X2 test (Lovell 1989). The levels for statistical significance were $P< 0.05$, $P< 0.01$ and $P<0.001$.

FIGURE 4. 6 : A SHOWING MONONUCLEATED CELL WITH MICRONUCLEUS
B SHOWING BINUCLEATED CELL WITH MICRONUCLEUS



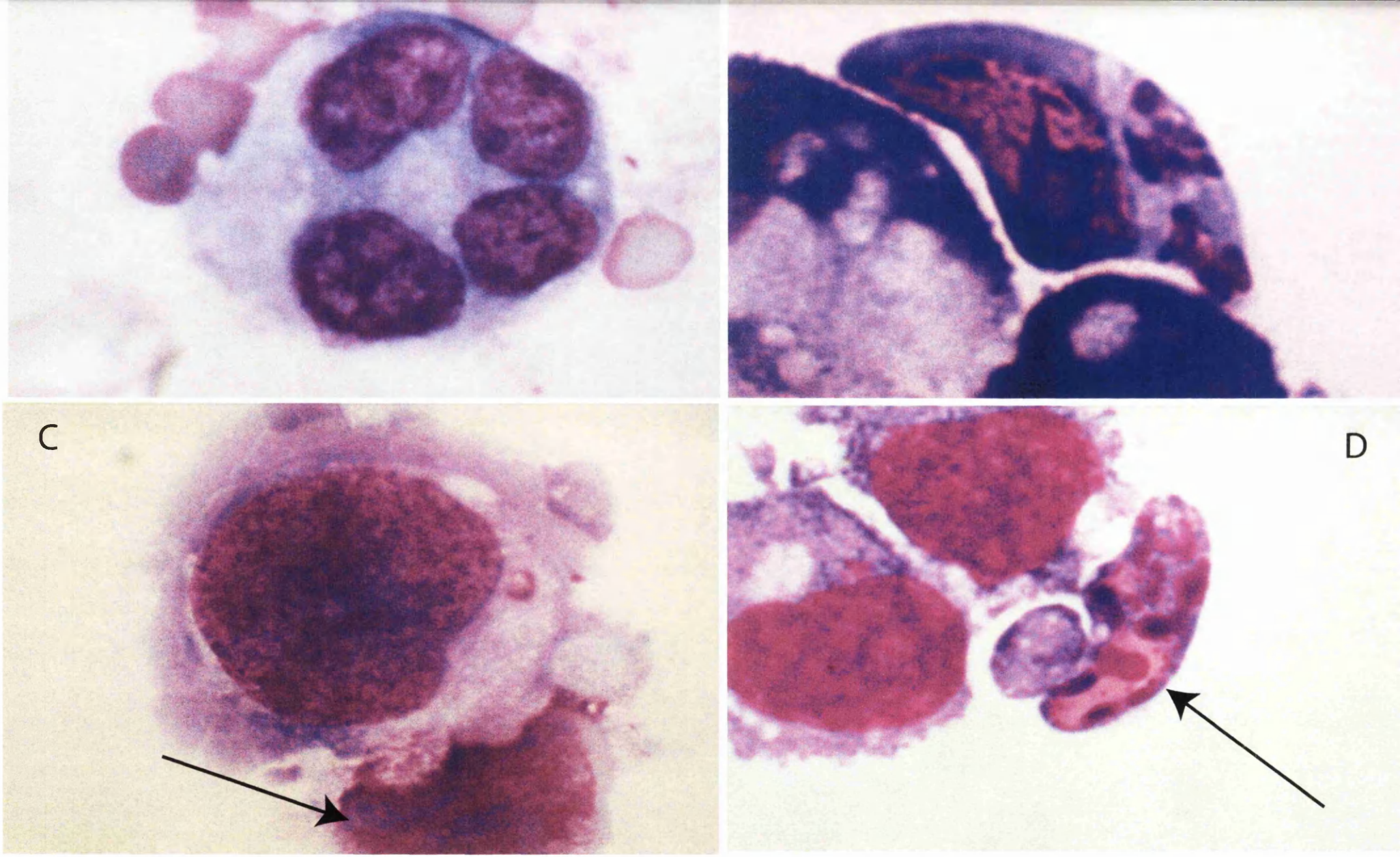
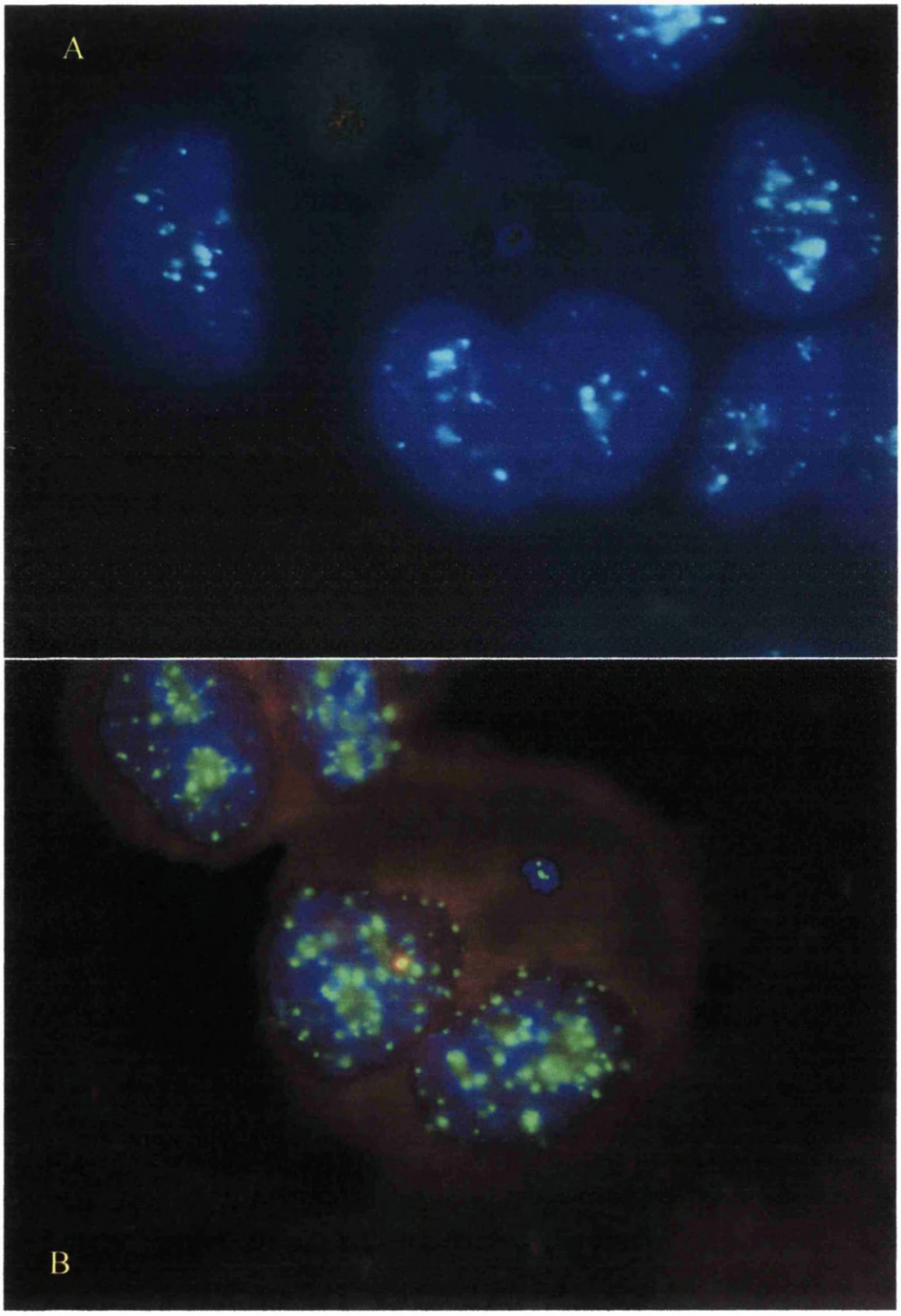


FIGURE 4.7 A. Multinucleated cell. B. Apoptotic cell C. Late stages of necrosis showing loss of the cytoplasm and cytoplasmic membrane with degeneration of the nucleus. D. Apoptotic cell showing completely broken down nuclear material within an intact cytoplasm and cytoplasmic membrane.

FGURE 4.8: SHOWING CREST STAINING. A. BINUCLEATE CELL WITH KINETOCHORE NEGATIVE MICRONUCLEUS. B. BINUCLEATE CELL WITH KINETOCHORE POSITIVE MICRONUCLEUS



4.3 RESULTS

The induction of micronuclei in the OE33 cells by the various bile acids were measured using the Cytokinesis Blocked Micronucleus Assay. The experiments with all the bile acids were performed in two different pH conditions (pH 7.3 and pH5.5). This was to mimic the in-vivo conditions as closely as possible. Although the physiological pH in the lower oesophagus in patients with GERD can vary from **4 to 7** (Kauer *et al.,* 1997), a pH lower than 5.5 was found to have a marked cytotoxic effect on the cell lines.

The bile acids were tested over a dose range of 10μmol to 3mmol. This range was selected based on the findings of Nehra *et al.*, 1999 and Iftikhar *et al*, 1993 who found that the concentration of various bile acids could vary in the range of ranging from 50μmol – 3mmol in patients with oesophageal reflux and Barrett's oesophagus. Deoxycholic and cholic acid were used in the range of 10μmol to 500μmol as concentrations of greater than 500μmol were found to be toxic to the cell lines.

4.31 MICRONUCLEI INDUCTION AT pH 7.3

Results of the OE33 Micronucleus assay with different bile acids at pH 7.3 are shown in the tables (For raw data vide appendix). Each table depicts the mean percentage of mononucleates, binucleates, multinucleates and MN in binucleate cells induced by different bile acids.

4.311 TAUROCHOLIC ACID

Data from this assay are presented in both tabular and graphical format (table 4.6 and figure 4.9). Taurocholic acid was used in the strength ranging from 100μmol to 3mmol. Concentrations above 3mmol were found to be extremely cytotoxic as seen

by complete necrosis and loss of integrity of the cells on slides. This prevented scoring of the cells.

Taurocholic acid induced a dose dependent reduction in the levels of binucleated cells. The percentage of the binucleate cell gradually decreased with increasing dose from 39.70% at 100μmol to 20.80% at 3mmol. Statistically significant reduction ($P<0.001$) in the proportion of the binucleate cells was seen at a concentration of 100μmol and above.

TCA did not show any statistically significant increases in Mn induction in binucleated cells. Maximum level of MN induction in binucleates of 1.16% was seen at concentration of 300μmol compared to 0.70% with control sample. There was no significant increase in the levels of multinucleated cells.

Table 4.7 and figure 4.10 show the results of apoptosis and necrosis. There was a progressive increase in the dose of the necrotic cells with increasing dose of TCA. The number of necrotic cells increased from 2.21% at 100μmol to 16.36% at 3000μmol. Statistically significant necrosis ($P<0.001$) was seen at the dose of 700mmol. There was no statistically significant change in the level of apoptosis in comparision with the control.

Table 4.6: Data for MN induction for Taurocholic acid treated OE33 cells

Dose in µmol/l	%Mono	%Binucleate	%Multinucleate	%MN Bn
00	54.29	44.80	0.94	0.70
100	59.50	39.70***	0.78	0.59
200	61.50	38.00***	0.53	0.99
300	66.60	32.80***	0.60	1.16
400	68.12	31.33***	0.56	0.79
500	64.66	34.70***	0.64	0.70
600	70.62	28.90***	0.49	0.50
700	67.32	32.00***	0.67	0.79
800	70.58	28.95***	0.47	0.45
900	71.00	28.43***	0.57	0.60
1000	76.45	23.00***	0.55	0.90
2000	76.17	23.33***	0.50	0.73
3000	78.57	20.80***	0.65	0.70

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%MNBn- frequency of binucleated cells containing micronuclei

Figure 4.9: MICRONUCLEUS INDUCTION BY TAUROCHOLIC ACID

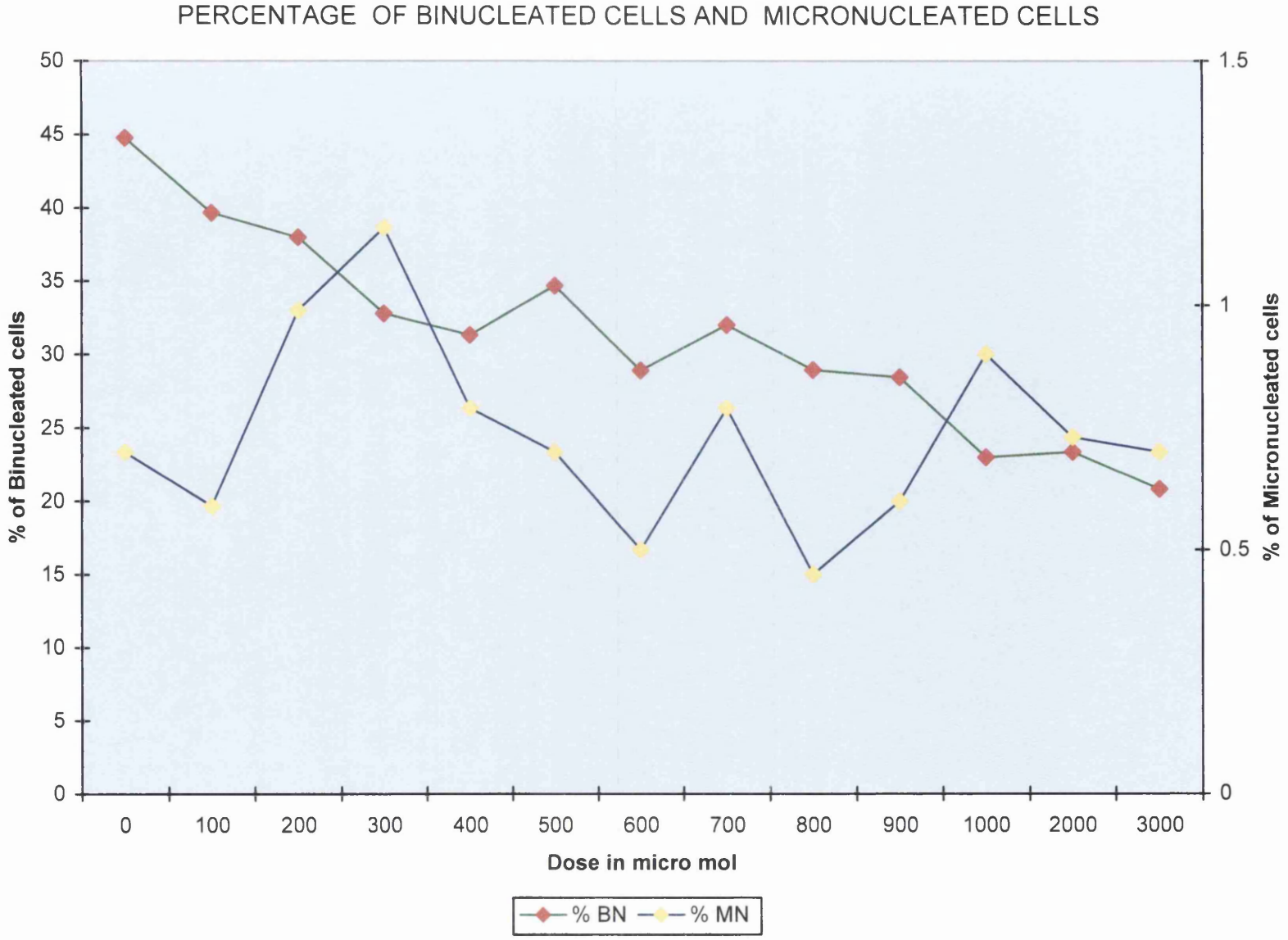
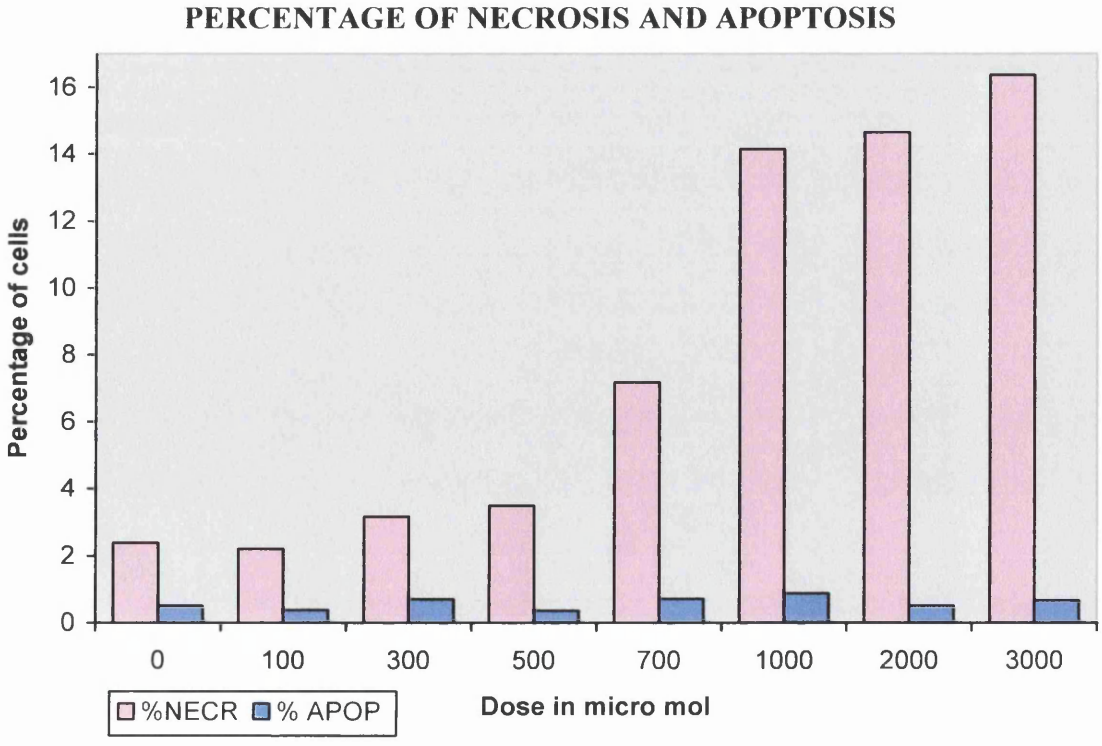


Table 4.7: Data for induction apoptosis and necrosis by Taurocholic acid

Dose in µmol/l	%Mono	%Binu	%Multi	%Apoptosis	%Necrosis
000	54.17	42.25	0.68	0.51	2.39
100	56.72	40.52	0.92	0.37	2.21
300	54.03	41.05	1.05	0.70	3.16
500	58.95	36.32	0.88	0.35	3.5
700	58.05	33.97	1.08	0.71	7.18***
1000	66.32	17.49	0.70	0.87	14.14***
2000	67.63	16.70	0.52	0.51	14.65***
3000	65.26	17.03	0.67	0.67	16.36***

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%apoptosis- frequency of apoptotic cells
%necrosis- frequency of necrotic cells

Figure 4.10: Induction of apoptosis and necrosis by taurocholic acid



4.312 GLYCOCHOLIC ACID

The results of MN induction in the OE33 cell lines by glycocholic acid, is shown in the table 4.8 and figure 4.11 and multinucleates along with micronucleated binucleate cells.

Glycocholic acid was used at concentration ranging from 100μmol to 3mmol. Concentrations above 3mmol were found to be extremely cytotoxic as seen by extensive necrosis.

There was dose dependent increase the levels of binucleated cells. The percentage of the binucleate cell gradually decreased from 43.30% at 100μmol to 20.61% at 3mmol while the proportion of mononucleated cells increased from 55.69% at 100μmol to 78.80% at 3mmol. A statistically significant decrease ($P<0.001$) in the level of binucleate cells was found at concentration of 100μmol and greater. The number of multinucleate cells did not show any statistically significant difference in comparision to the control.

The induction of MN in binucleate cells did not show any statistically significant increase with increasing dose of GCA. The MN induction in binucleate cells was 0.80% in the control samples whereas a maximum of 1.24% was observed at 2000mmol.

Table 4.9 and figure 4.14 show the results of apoptosis and necrosis. There was a dose dependent increase in the number of necrotic cells. The level of necrotic cells increased from 2.95% at 100μmol to 16.13% at 3000μmol. Statistically significant necrosis ($p=0.05$) was seen at the dose of 500μmol and above.

A maximum level of apoptosis of 1.1% was seen at doses of 1 and 2 mmol compared to a level of 0.70% in the control samples. There was no statistically significant increase in the level of apoptosis.

Table 4.8: Data for MN induction for Glycocholic acid treated OE33 cells

Dose in µmol/l	%Mono	%Binuc	%Multinucleate	%MN BN
00	49.0	50.0	0.99	0.80
100	55.69	43.30***	1.03	0.60
200	64.99	34.40***	0.59	1.20
300	66.52	32.83***	0.65	1.10
400	64.74	34.50***	0.58	0.93
500	70.20	29.11***	0.69	0.98
600	67.35	32.00***	0.65	0.69
700	68.43	31.00***	0.57	0.80
800	71.59	27.73***	0.69	0.79
900	67.98	31.50***	0.54	0.90
1000	69.64	29.77***	0.59	0.80
2000	76.35	23.05***	0.59	1.24
3000	78.80	20.61***	0.58	0.85

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%MNBn- frequency of binucleated cells containing micronuclei

Figure 4.11: MICRONUCLEUS INDUCTION BY GLYCOCHOLIC ACID

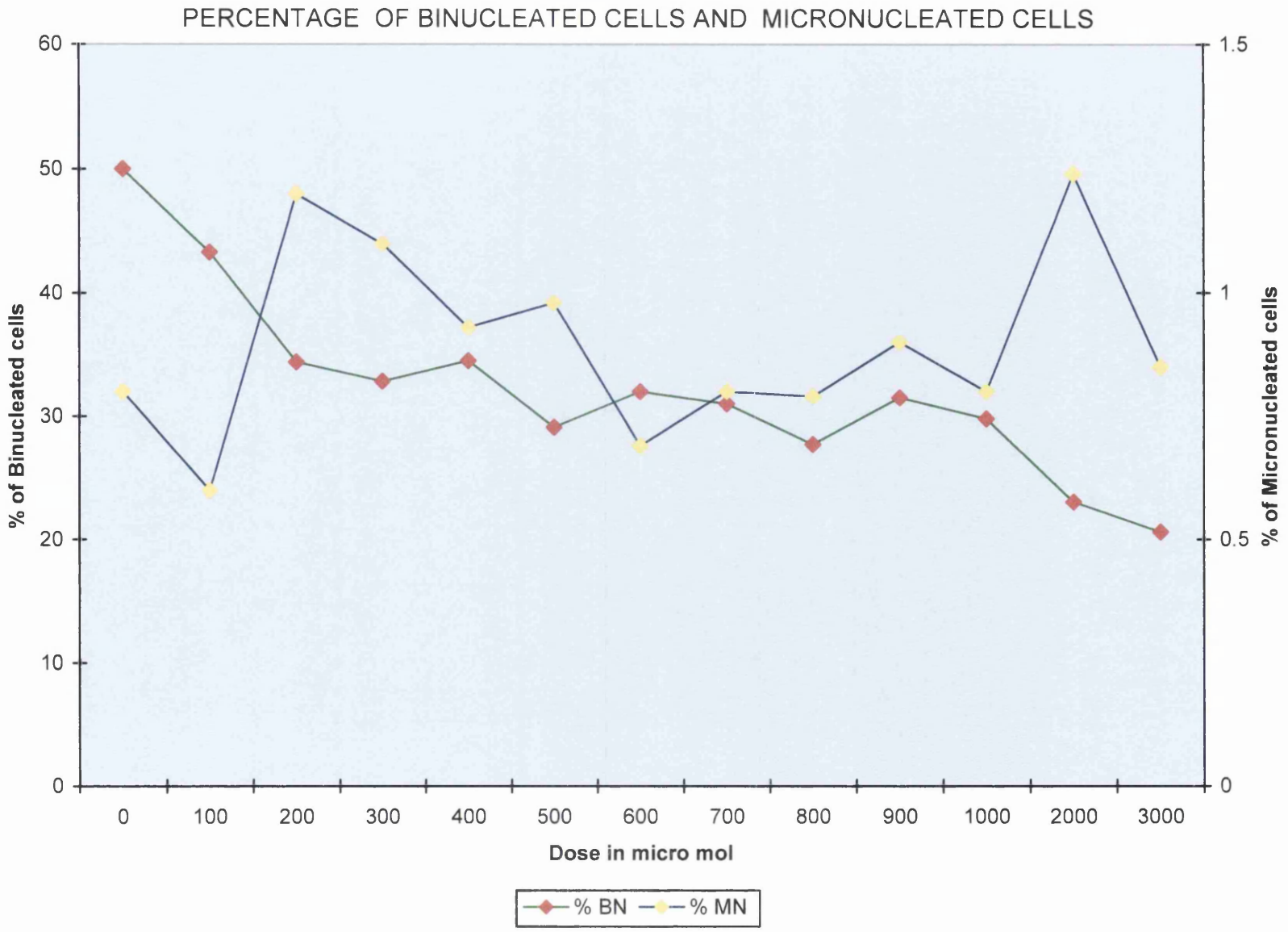
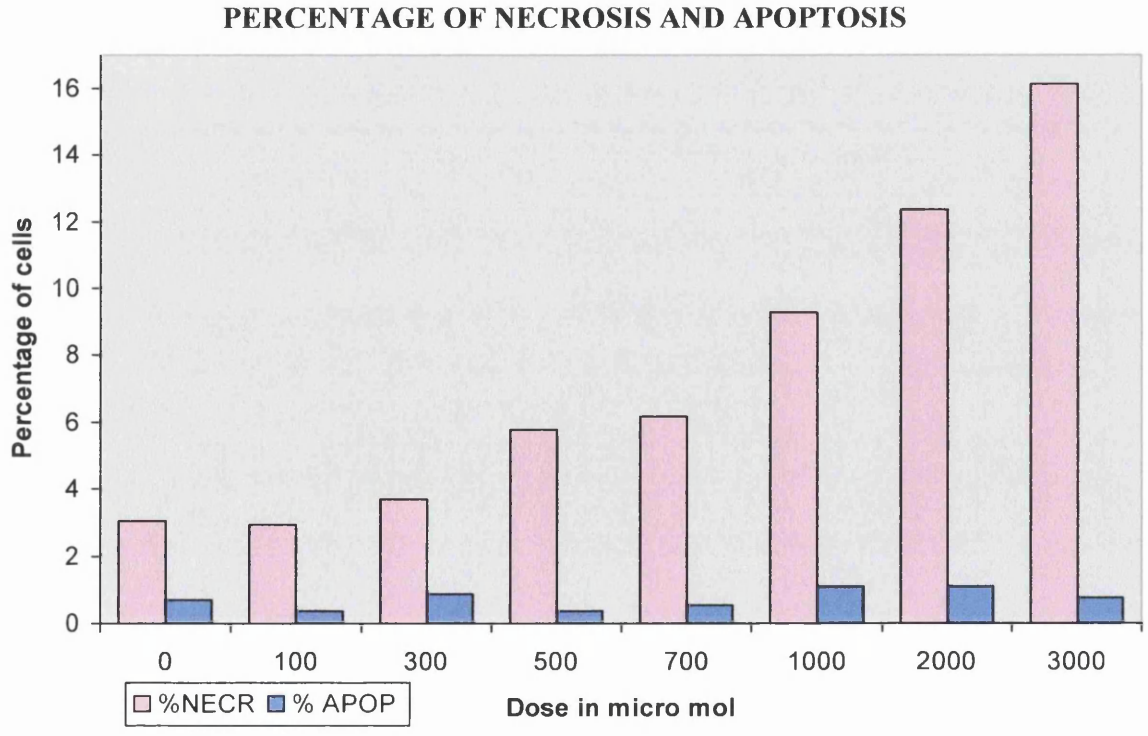


Table 4.9: Data for induction apoptosis and necrosis by glycocholic acid

Dose in µmol/l	%Mono	%Binu	%Multi	%Apoptosis	%Necrosis
000	46.51	49.02	0.72	0.70	3.04
100	50.92	45.20	0.55	0.37	2.95
300	45.05	49.47	083	0.88	3.71
500	51.71	41.44	0.72	0.36	5.76*
700	58.08	34.30	0.91	0.54	6.17*
1000	68.46	20.41	0.92	1.1	9.27***
2000	62.55	23.43	0.55	1.1	12.36***
3000	57.99	24.35	0.77	0.76	16.13***

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%apoptosis- frequency of apoptotic cells
%necrosis- frequency of necrotic cells

Figure 4.12: Induction of apoptosis and necrosis by glycocholic acid



4.313 TAURODEOXYCHOLIC ACID

Table 4.10 (for raw data see appendix) shows the results of MN induction in the OE33 cell lines by taurodeoxycholic acid. The means of frequencies of mono-, bi- and multinucleates along with micronucleated binucleate cells are shown in the table. Taurodeoxycholic acid was used in the highest concentration of 2mmol/l. Concentration greater than 2mmol/l induced extensive cytotoxicity.

TDCA induced cytotoxicity in a dose dependent manner. There was a progressive decrease in the level of binucleated cells with increasing dose of TDCA (figure 4.13). The percentage of the binucleate cells decreased from 45.89% at 100μmol to 22.10% at 2mmol while the proportion of mononucleated cells increased from 53.24% at 100μmol to 77.52% at 2mmol. A statistically significant decrease ($P<0.001$) in the level of binucleate cells was found at concentration of 600μmol and above. There was no statistically significant difference in the number of multinucleate cells between the various doses and the control.

The induction of MN was the maximum at doses between 400 to 700μmol/l with a level of 1.1% compared to 0.79% with the control. None of the doses of TDCA showed statistically significant induction of MN in the binucleate cells.

The results of apoptosis and necrosis are shown in the table 4.11 and figure 4.14. Necrosis induced by TDCA was dose dependent. The level of necrotic cells increased from 1.54% at 100μmol/l to 20.82% at 1000mmol/l (figure 4.14). Statistically significant necrosis ($p<0.05$) was seen at the dose of 300μmol and above.

A maximum level of apoptosis of 1.50% was seen at 700μmol/l compared to a level of 0.83% in the control samples. TDCA did not show statistically significant induction of apoptosis at the various tested doses.

Table 4.10: Data for MN induction for Taurodeoxycholic acid treated OE33 cells

Dose in µmol/l	%Mono	%Binuc	%Multinucleate	%MN BN
00	53.96	45.28	0.76	0.79
100	53.24	45.89	0.92	0.70
200	54.27	45.10	0.66	0.49
300	50.09	49.16	0.74	0.70
400	56.06	43.2	0.78	1.10
500	54.15	44.90	0.99	1.01
600	64.22	35.28***	0.45	0.91
700	59.19	40.10***	0.75	1.10
800	66.57	33.00***	0.46	0.70
900	70.22	29.21***	0.57	0.69
1000	68.50	30.60***	0.896	0.81
2000	77.52	22.10***	0.39	0.58

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%MNBn- frequency of binucleated cells containing micronuclei

FIGURE 4.13: MICRONUCLEUS INDUCTION BY TAURODEOXYCHOLIC ACID

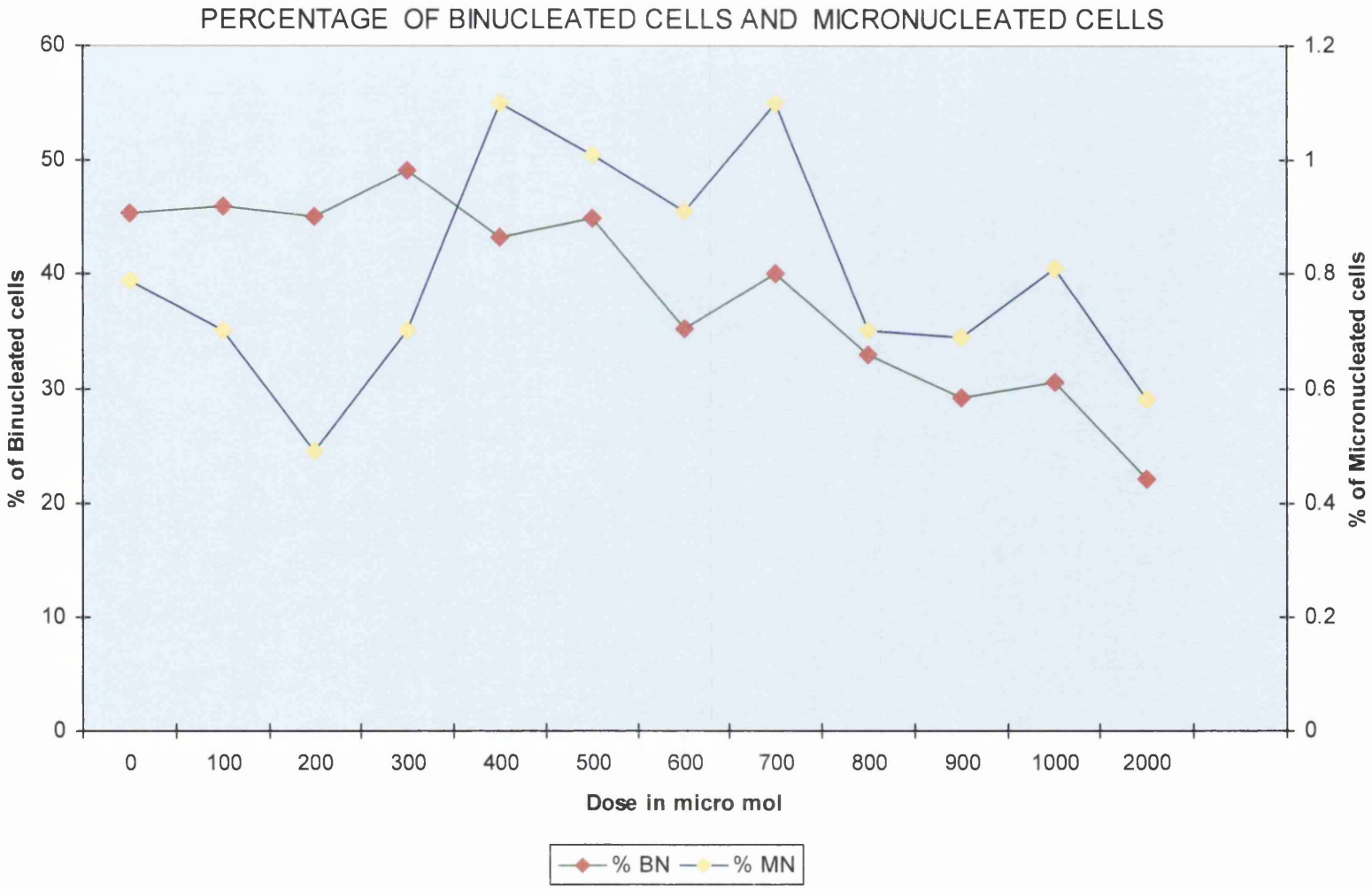
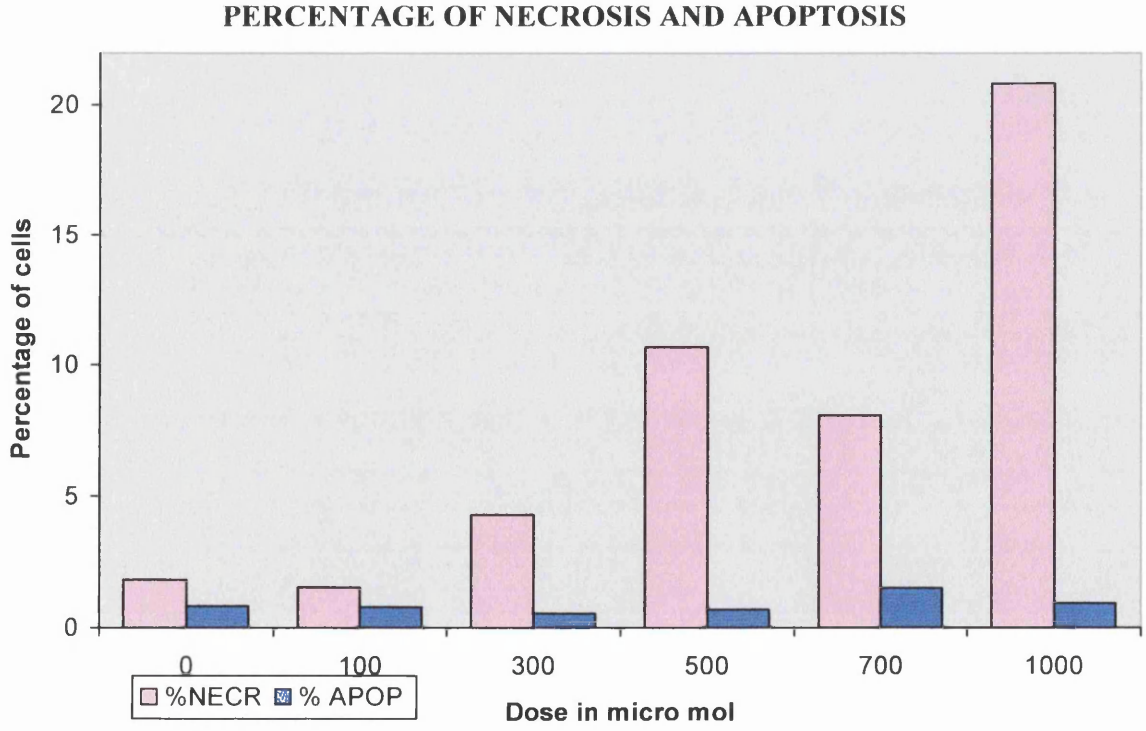


Table 4.11: Data for induction apoptosis and necrosis by Taurodeoxycholic acid

Dose in µmol/l	%Mono	%Binu	%Multi	%Apoptosis	%Necrosis
000	49.84	46.87	0.66	0.83	1.82
100	51.15	45.77	0.77	0.77	1.54
300	51.29	43.20	0.69	0.52	4.30*
500	53.33	34.33	1.00	0.67	10.7***
700	56.72	32.84	00.90	1.50	8.10***
1000	60.04	35.46	1.50	0.94	20.82***

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%apoptosis- frequency of apoptotic cells
%necrosis- frequency of necrotic cells

Figure 4.14: Induction Of Apoptosis And Necrosis By Taurodeoxycholic Acid



4.314 GLYCODEOXYCHOLIC ACID

The results of MN analysis with GDCA are shown in the table 4.12 and figure 4.15 (for raw data see appendix). GDCA was used in the concentration ranging from 100μmol to 2000μmol/l. Concentrations of greater than 2000μmol induced extensive necrosis as seen by the loss of integrity of the cells under simple microscope.

Induction of cytotoxicity by GDCA was dependent on the dose. There was a gradual reduction of the binucleate cells with the increasing dose of GDCA.

The percentage of the binucleate cells decreased from 47.96% at 100μmol/L to 30.60% at 2000μmol/L. Similarly the level of mononucleated cells increased from 51.08% at 100μmol/L to 69.10% at 2000μmol/ml. A statistically significant decrease ($P<0.001$) in the level of binucleate cells was found at concentration of 200μmol/L. The number of multinucleate cells seemed to decrease at high dose of GDCA though this was not statistically significant.

The induction of MN in binucleate cells by GDCA was not much different from that observed in the control samples. None of the doses of the GDCA showed significant induction of MN.

The results of apoptosis and necrosis are shown in the table 4.13 and figure 4.16. GDCA induced necrosis in a dose dependent manner. The level of necrotic cells increased from 1.93% at 100μmol/L to 17.67% at 1000μmol/L. Statistically significant necrosis ($p<0.001$) was seen at the dose of 500μmol and above. A maximum level of apoptosis of 1.73% was seen at 500μmol/l compared to a level of 0.94% in the control samples. Induction of apoptosis by various doses of GDCA was not found to be statistically significant.

Table 4.12: Data for MN induction for Glycodeoxycholic acid treated OE3 3 cells

Dose in µmol/l	%Mono	%Binuc	%Multinucleate	%MN BN
00	51.72	47.29	0.99	0.83
100	51.08	47.96	0.96	0.63
200	58.77	40.77***	0.46	0.62
300	57.95	41.77***	0.30	0.71
400	60.68	38.97***	0.35	0.70
500	60.91	38.79***	0.31	0.69
600	62.50	37.14***	0.37	0.59
800	63.50	36.09***	0.42	0.84
1000	69.20	30.50***	0.30	0.59
2000	69.10	30.60***	0.28	0.71

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%MNBn- frequency of binucleated cells containing micronuclei

FIGURE 4.15: MICRONUCLEUS INDUCTION BY GLYCODEOXYCHOLIC ACID

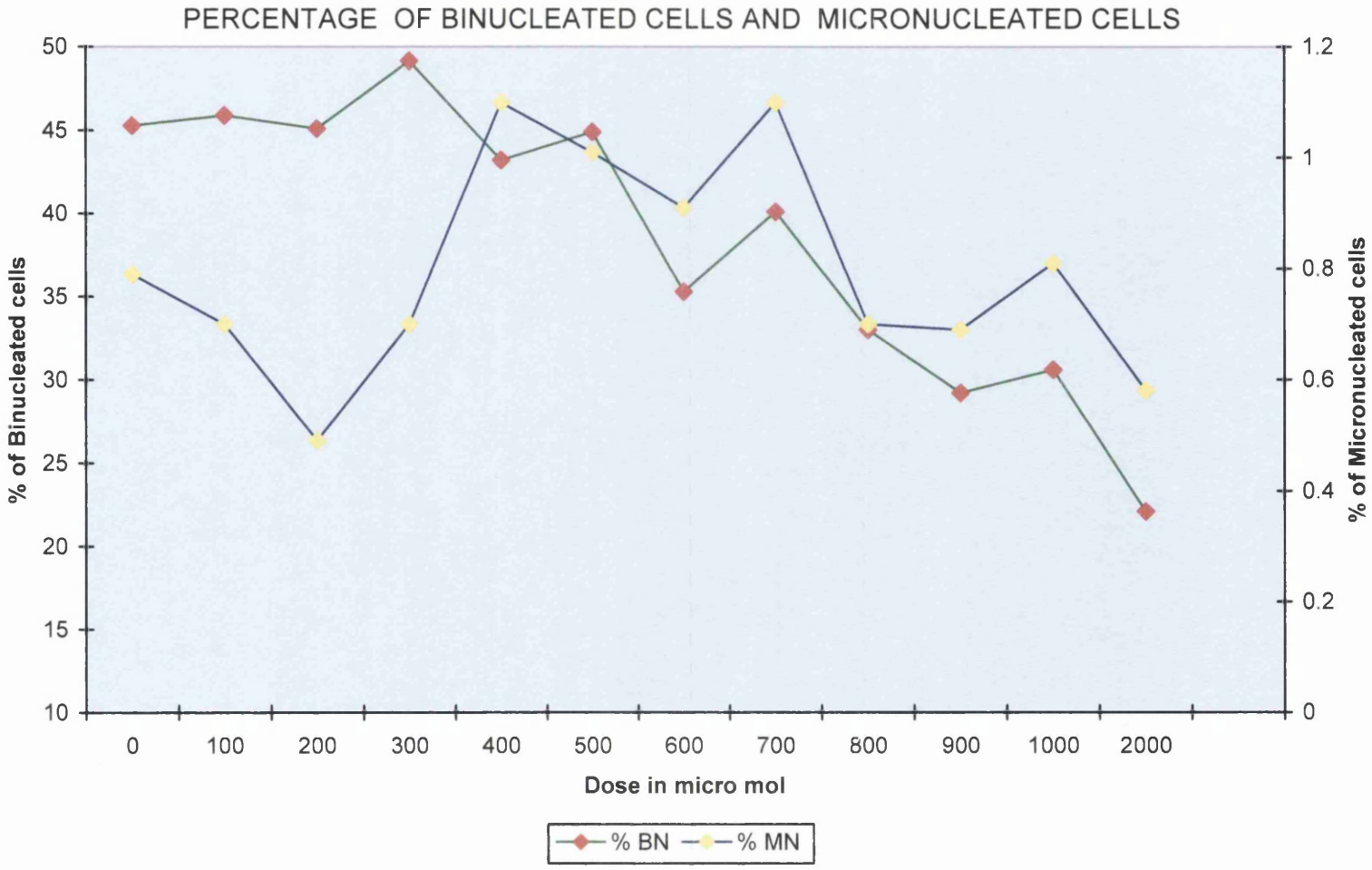
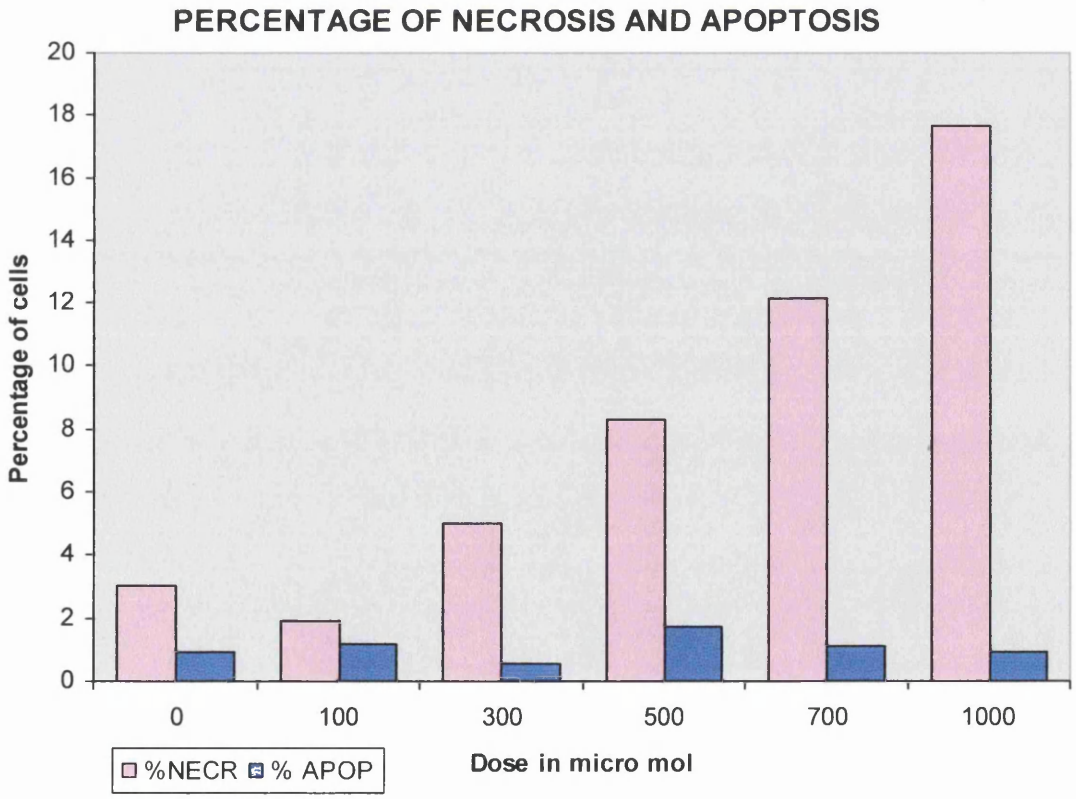


Table 4.13 Data for induction apoptosis and necrosis by Glycodeoxycholic acid

Dose in µmol/l	%Mono	%Binu	%Multi	%Apoptosis	%Necrosis
000	47.08	44.82	0.75	0.94	3.01
100	48.36	47.58	0.97	1.16	1.93
300	51.52	42.25	0.71	0.53	4.99
500	51.12	38.00	0.86	1.73	8.29***
700	51.38	34.31	1.1	1.10	12.11***
1000	50.47	29.97	0.95	0.95	17.67***

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%apoptosis- frequency of apoptotic cells
%necrosis- frequency of necrotic cells

FIGURE 4.16: Induction Of Apoptosis And Necrosis By Glycodeoxycholic Acid



4.315 DEOXYCHOLIC ACID

The results of MN analysis with DCA are shown in the table 4.14 and figure 4.17 (for raw data see appendix). The concentrations tested were 10, 25, 50, 100, 200, 300 and 400μmol/l. Unlike the conjugated bile acids concentrations of 500μmol and above showed extensive cytotoxicity as seen by complete necrosis of the cells under light microscopy.

Table figure 4.17 shows that DCA induced cytotoxicity in a dose dependent manner. The proportion of binucleate cells gradually decreased from 36% at 10μmol to 11% at the highest dose of 400μmol/L. The decrease in proportion of the binucleate cells was statistically significant ($P<0.001$) at concentrations of 10μmol and above. The level of multinucleate cells did not vary significantly in comparison with the control.

At concentrations of 50, 100 and 200 there was a statistically significant ($p<0.05$) increase in the induction of MN in binucleated cells. At concentration of 100, 200 and 400μmol the the frequency of MN induction was more than three times the control value. In order to further investigate the content and of MN and the mechanism of induction of MN by DCA, CREST analysis was performed. The CREST analysis was performed using the concentrations (50, 100 and 200μmol) which induced statistically significant number of micronuclei in binucleate cells. The results of kinetochore analysis are presented in the table 4.15 and figure 4.18.

The kinetochore analysis showed that the MN were predominantly kinetochore negative i.e, most of the micronuclei did not exhibit a fluorescence signal. The proportion of kinetochore positive MN in the DCA treated slides was much lower than that seen in the control indicating a clastogenic effect. The proportion of kinetochore negative micronuclei increased with increasing concentration of DCA (67% at 50μmol to 76% at 200μmol). Hence the mechanism of induction of MN by DCA was mostly clastogenic.

Table 4.16 and figure 4.19 show the results of apoptosis and necrosis induced by DCA. DCA induced necrosis in a dose dependent manner. The level of necrotic cells increased from 2.63% at 10μmol/L to 15.98% at 1000μmol/L. Statistically significant necrosis ($p<0.001$) was seen at the dose of 50μmol and above. The induction of apoptosis by DCA was the highest at the concentrations between 50 and 300μmol/L. At concentrations of 100μmol/L the level of induction of apoptosis was almost six times the control. The induction of apoptosis by DCA was found to be statistically significant.

Table 4.14: Data for MN Induction for Deoxycholic Acid treated OE3 3 Cells

Dose in μmol/l	%Mono	%Binuc	%Multinucleate	%MN BN
00	55.61	43.66	0.74	0.65
10	63.36	36.00***	0.67	0.97
25	59.69	39.65***	0.67	1.19
50	65.46	33.70***	0.84	1.80*
100	69.89	29.44***	0.67	2.10**
200	79.22	20.0***	0.76	2.02*
300	81.60	17.90***	0.49	1.52
400	88.85	11.00***	0.56	2.2*

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%MNBn- frequency of binucleated cells containing micronuclei

FIGURE 4.17: MICRONUCLEUS INDUCTION BY DEOXYCHOLIC ACID

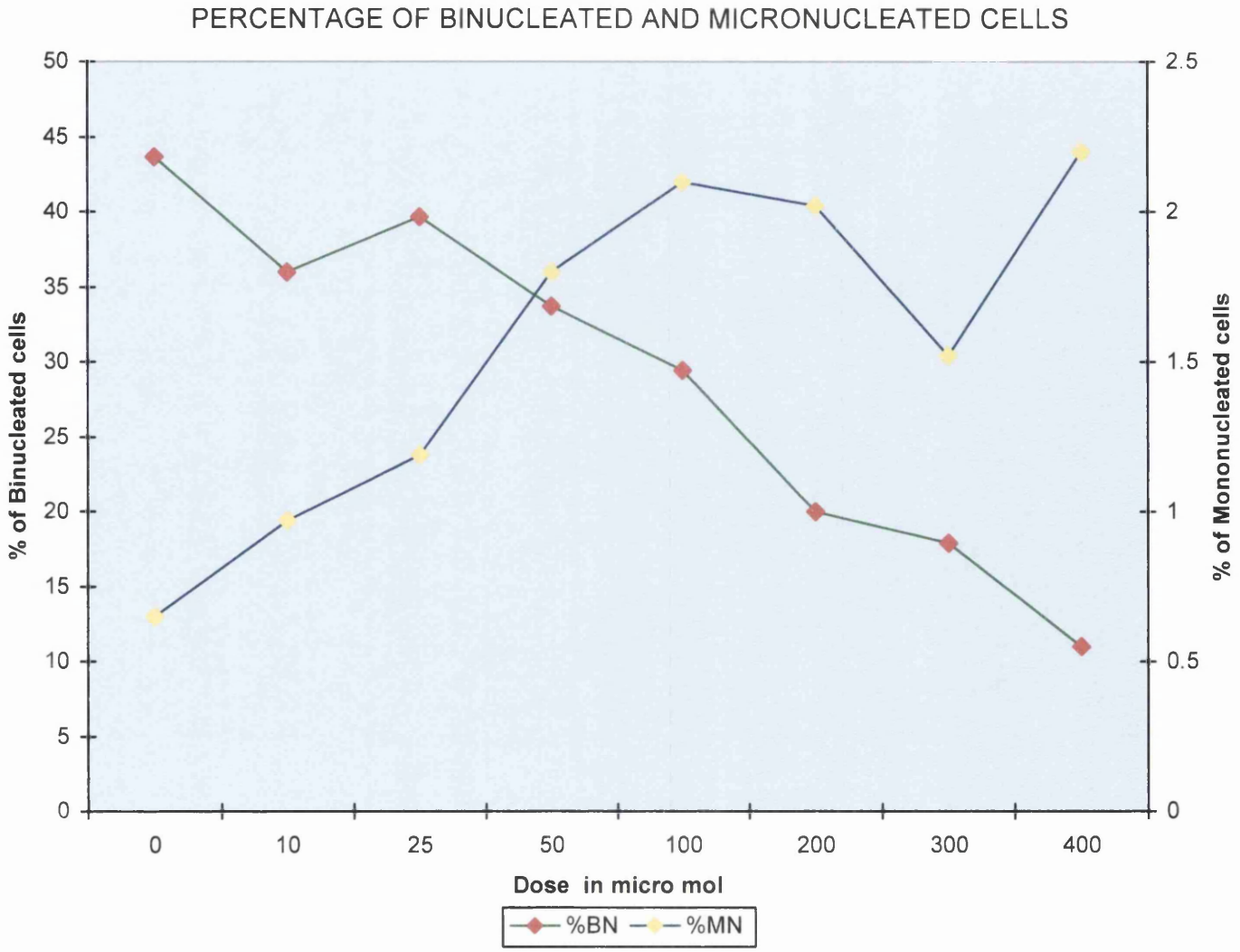


Table 4.17: Data for MN Induction for Cholic Acid treated Oe33 Cells

Dose in µmol/l	%Mono	%Binuc	%Multinucleate	%MN BN
00	49.81	49.50	0.73	0.79
10	52.04	47.00	0.99	0.60
25	59.43	40.00***	0.55	0.99
50	59.70	39.70***	0.64	0.71
100	55.94	43.42***	0.64	0.69
200	63.99	35.30***	0.71	1.00
300	66.19	33.20***	0.66	1.20
400	74.79	24.65***	0.55	0.67
500	82.36	17.00***	0.67	0.79

P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%MNBn- frequency of binucleated cells containing micronuclei

Figure 4.20: MICRONUCLEUS INDUCTION BY CHOLIC ACID

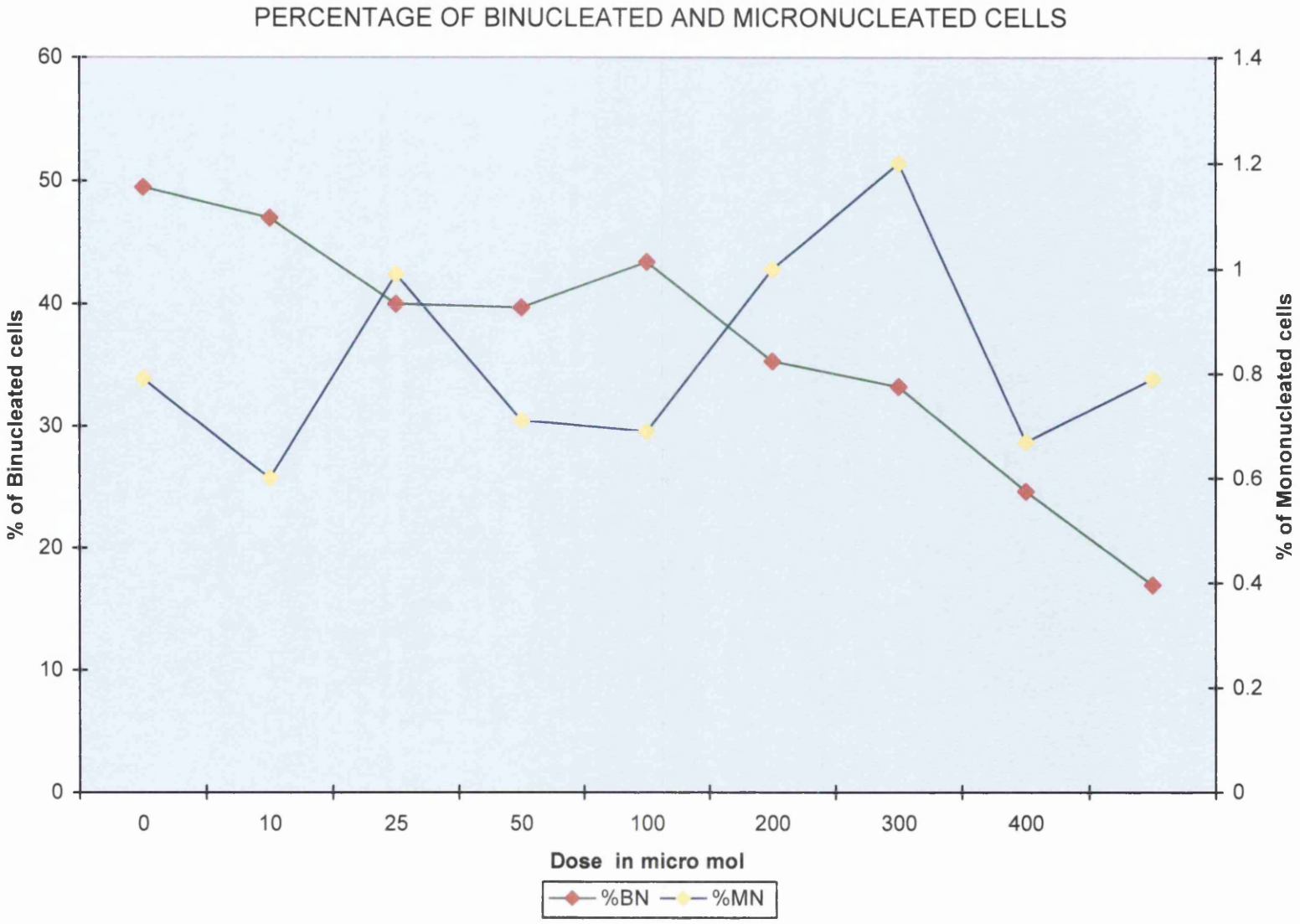


Table4.18: Data for Induction Apoptosis and Necrosis by Cholic Acid

Dose in µmol/l	%Mono	%Binu	%Multi	%Apoptosis	%Necrosis
000	46.77	48.80	0.55	0.74	3.14
10	45.86	49.72	0.92	0.74	2.76
25	49.90	45.49	1.15	0.97	2.50
50	51.09	43.82	1.09	0.36	3.64
100	50.18	40.26	1.47	1.47	6.62*
200	54.81	35.93	0.73	1.82	6.72**
300	71.57	19.80	1.18	0.98	6.47*
400	61.97	23.94	1.01	0.88	12.32***
500	67.19	20.73	0.69	0.52	10.88***

*P<0.05 **P<0.01 ***P<0.001

%Mono- frequency of mononucleated cells

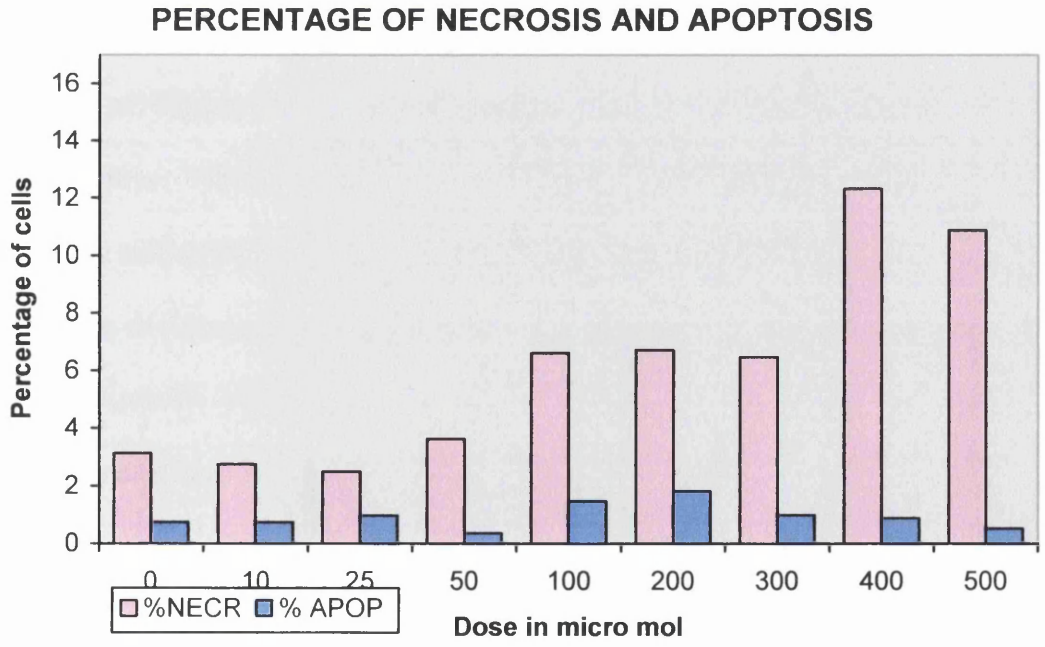
%Binucleate- frequency of binucleated cells

%Multinucleate- frequency of cells containing more than 2 nuclei

%apoptosis- frequency of apoptotic cells

%necrosis- frequency of necrotic cells

Figure 4.21: Induction Of Apoptosis And Necrosis By Cholic Acid



4.317 MITOMYCIN C

Mitomycin C was used as a positive control. Mitomycin C is a known clastogen. MMC both alklylates and cross links DNA, which leads to breakage of DNA. MMC was used in the concentration 10, 30, 50, 70 and 100 μmols/L.

The data of MN induction by various concentrations of MMC is shown in the table 4.19 and figure 4.22. MMC induced cytotoxicity which was indicated by a decrease in the level of binucleate cells with an increasing concentration. The proportion of bineucletate cells decreased from 50.10% at 10μmol/L to 33.53% at 100μmol/L. The highest level of microneucleus was induced at concentrations 70 μmol/L and 100μmol/L with 4.17% and 3.70% respectively compared to 0.73% with control.

To further evaluate the mechanism of induction of microneuclei kinetocore staining (CREST analysis) was performed table 4.20 and figure 4.23 show the results of CREST analysis of mitomycin C using concentrations of 30, 50 and 70 μmol/L. The kinetocore analysis showed that the microneulei were predominantly kinetocore negative. The proportion of k- to k+ was greater with mitomycin C compared to the control. At concentrations of 50μmol/L and 70μmol/L greater than 75% of the microneuclei were kinetocore negative. Table 4.21 and figure 4.24 show the results of induction of apoptosis and necrosis by mitomycin C in the OE33 cells. The level of necrosis increased from 2.76 at concentration of 10μmol/L to 6.62% at 100μmol/L. The highest level of apoptosis was induced at 100μmol/L which was almost twice the level seen in control (1.47% versus 0.74%). The induction of apoptosis by mitomycin C was not found to be statistically significant.

Table 4.19: Data for MN induction for Mitomycin C treated OE33 cells

Dose in µmol/l	%Mono	%Binuc	%Multinucleate	%MN BN
00	51.88	47.64	0.48	.73
10	49.19	50.10**	0.74	1.08
30	56.35	42.90***	0.80	2.15*
50	61.49	37.82**	0.69	3.95***
70	55.37	44.91*	0.60	4.17***
100	65.96	33.53***	0.51	3.70***

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%MNBn- frequency of binucleated cells containing micronuclei

Figure 4.22: MICRONUCLEUS INDUCTION BY MITOMYCIN C

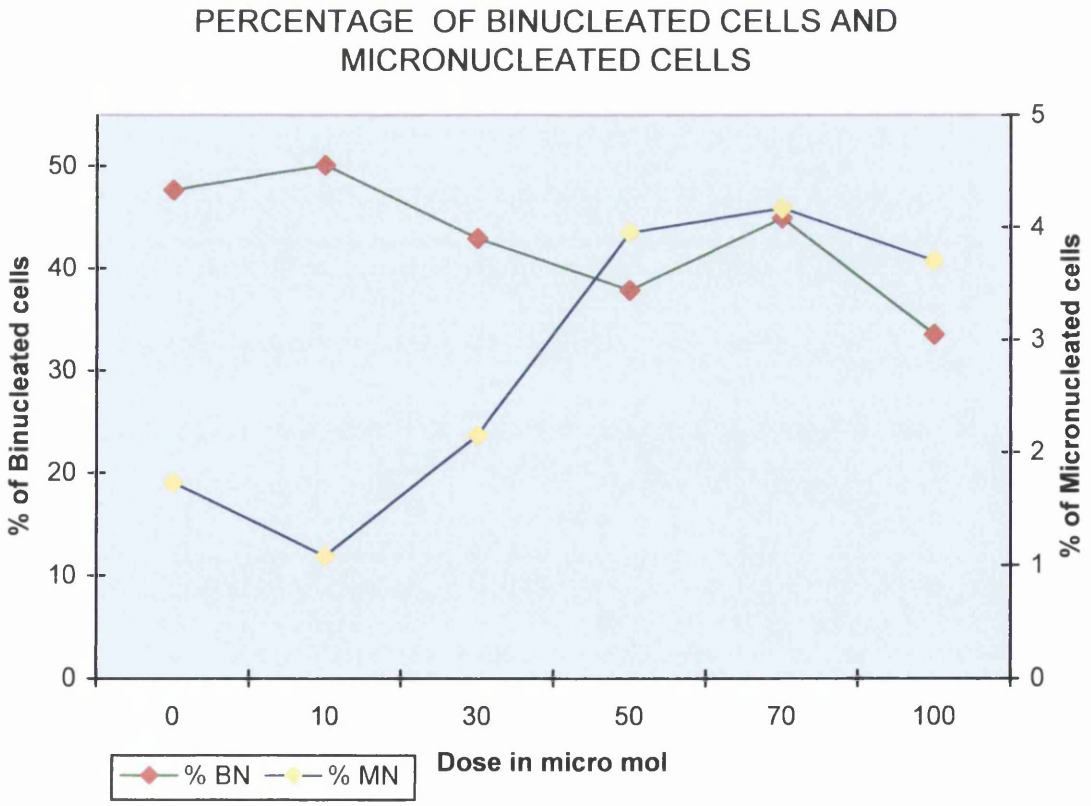


TABLE 4.20: SHOWING PROPORTION OF KINETOCHORE POSITIVE AND KINETOCHORE NEGATIVE MICRONUCLEI INDUCED BY MITOMYCIN C

Dose ηg/ml	K+ Mn	K- Mn	Total Mn	Proportion Of K+	Proportion Of K-	%MN Bn	%Mnbn K+$^{\Psi}$	%Mnbn K-$^{\Psi}$
00	24	40	64	0.37	0.63	.73	0.27	0.46
30	28	58	86	0.33	0.67	2.15	0.71	1.44
50	23	77	100	0.23	0.77	3.95	0.91	3.04
70	19	81	100	0.19	0.81	4.17	0.79	3.38

$^{\Psi}$%MNBn K+ and %MNBn K- calculated by multiplying %MNBn by proportion of MN K+ or K-

Figure 4.23: FREQUENCIES OF KINETOCHORE POSITIVE AND KINETOCHORE NEGATIVE MICRONUCLEI IN BINUCLEATE CELLS INDUCED BY MITOMYCIN C

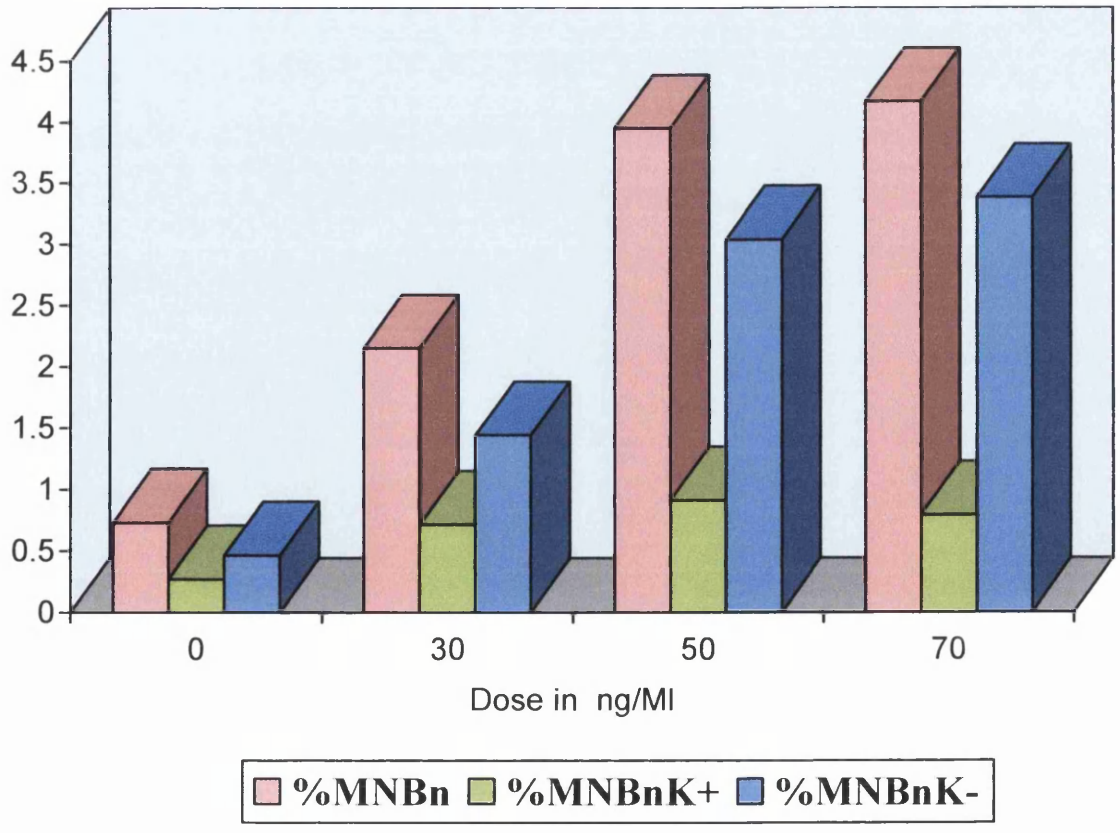
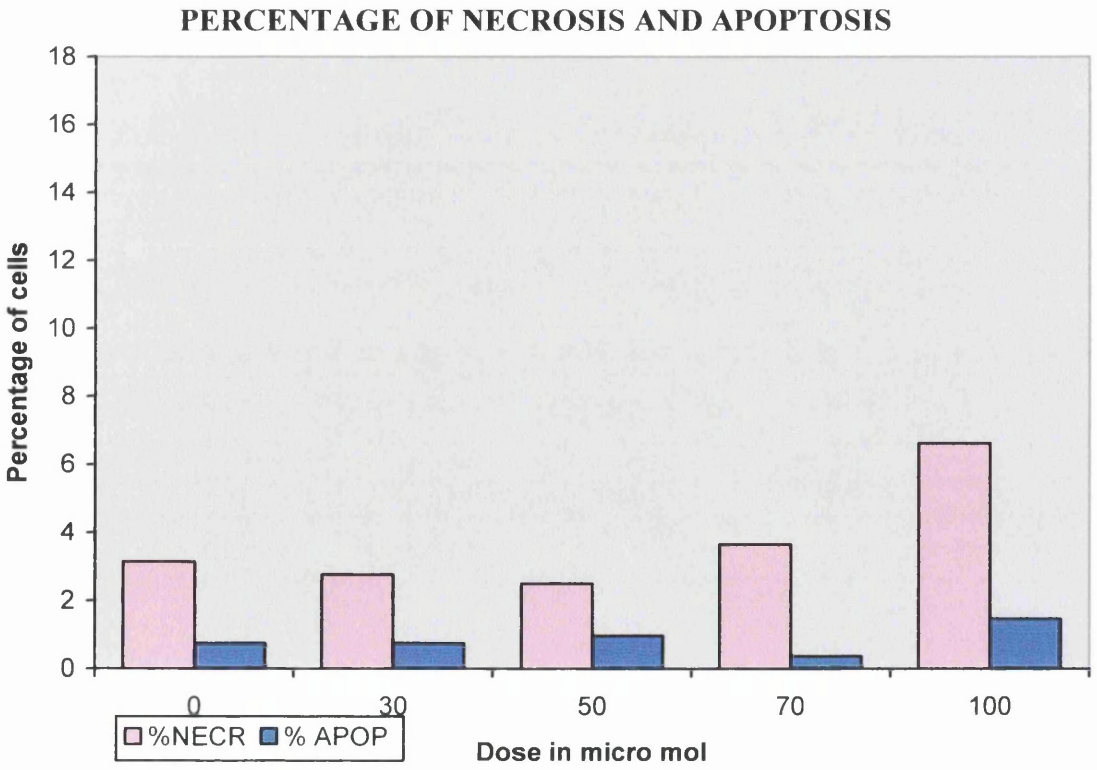


Table 4.21: Data for Induction Apoptosis and Necrosis by Mitomycin C

Dose in µmol/l	%Mono	%Binu	%Multi	%Apoptosis	%Necrosis
00	46.77	48.80	0.55	0.74	3.14
10	45.86	49.72	0.92	0.74	2.76
50	49.90	45.49	1.15	0.97	2.50
70	51.09	43.82	1.09	0.36	3.64
100	50.18	40.26	1.47	1.47	6.62*

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%apoptosis- frequency of apoptotic cells
%necrosis- frequency of necrotic cells

Figure 4.24: Induction Of Apoptosis And Necrosis By Mitomycin **C**



4.32 MICRONUCLEI INDUCTION AT PH 5.5

The second experiment evaluated the effect of bile acids at pH 5.5. Treatment with bile acids, cytochalasin-B and harvesting of the cells were all performed in a similar manner as previous experiments. The results of various treatments with bile acids at pH 5.5 are shown in the table from 4.22 to table 4.33 (For raw data vide appendix). Each table depicts the mean percentage of mono-nucleates, binucleates, multinucleates and MN in binucleate cells induced by different bile acids.

TCA, GCA, TDCA and GDCA were used in the concentrations of 100, 500 and 1000μmol/L. DCA was used in the concentration of 50, 100 and 200 μmol/L, while CA was used in the concentration of 50, 100, 200 and 500μmol/L.

Treatments with all bile acids induced a greater degree of cytotoxicity in comparision to similar strengths at neutral pH. Concentrations of TCA, GCA, TDCA and GDCA of greater than 1000 μmol/L induced generalized cytotoxicity. DCA induced generalized cytotoxocity at concentrations above 200μmol/L whilst CA induced generalized cytotoxicity at concentrations greater than 500μmol/L. With all bile acids there was a greater dose related decrease in percentage of bineucleate cells at pH 5.5 compared to pH 7.3.

Figures 4.25, 4.27, 4.29 and 4.31 show the data from MN analysis of TCA, GCA, TDCA and GDCA. The proportion of binucleate cells decreased with increasing dose of bile acids, the highest proportion of binucleates being seen at 100μmol and the least at 1000μmol. The proportion of binucleates in acidic control (pH 5.5) was much lower than that of neutral control (pH 7.3) indicating a greater degree cytotoxicity and inhibition of cellular division. The overall induction of MN in the binucleates with all the four bile acids (TCA, GCA, TDCA and GDCA) was relatively higher at acidic pH than at neutral pH. The difference was not statistically significant. Similarly the degree of apoptosis and necrosis was higher at pH 5.5 for all strengths of bile acids.

The difference in the level of necrosis at the two different pH conditions was found to be statistically significant. Though there was relatively increased apoptosis in the acidic medium the difference was not statistically significant (see table 4.23, 4.25, 4.27, 4.29 and figure 4.26, 4.28, 4.30 and 4.32).

Table 4.30 and figure 4.33 show the data from MN analysis of DCA at pH 5.5.

DCA induced a higher degree of cytotoxicity at acidic pH. Concentration of greater than 200μmol induced extensive necrosis. There was rapid fall in the proportion of binucleate cells from 36.82% with control to 10.19 at concentrations of 200μmol. The level of induction of MN in binucleates increased to almost three times the levels of control at concentrations of 200μmol/L (2.88% vs 0.96). Due to greater degree of cytotoxicity at concentrations of 100 and 200mmol the number of binucleate cells counted were relatively small (around 500 binucleate cells were counted). Though there is increase in the number of micronuclei this has to be interpreted with caution due to the high degree of cytotoxicity at these concentrations.

Table 4.31 and figure 4.34 shows the induction of apoptosis and necrosis by DCA at pH of 5.5 at concentrations of 100 and 200mmol the proportion of necrotic cells was greater than 30% and more than 40% of the remaining cells were mononucleate. The degree of apoptosis induced by DCA was almost three times the control level at the two highest concentrations (2.97 and 2.70 vs 1.18).

Table 4.32 and figure 4.35 show the data from MN analysis of CA at pH 5.5. CA was used in the concentration of 50 – 500μmol/L. The proportionate of binucleate cells fell from 32.49% at 50μmol to 8.9% at 500μmol/L. The induction of MN was highest at 500μmol/L (nearly twice the control level). Due to extensive degree of cytotoxicity at this concentration the number of binucleated cells counted was around 500.

At higher concentrations the level of apoptosis induced by CA was almost twice the control levels (2.18% vs 1.38%) although this was not found to be statistically significant (table 4.33 and figure 4.36).

Table 4.22: Data for MN induction for Taurocholic acid treated OE3 3 cells at **pH 5.5**

Dose in µmol/l	%Mono	%Binucleate	%Multinucleate	%MN Bn
00	65.679	34.03	0.29	0.95
100	64.89	34.81	0.30	0.80
500	73.43	26.32***	0.25	1.00
1000	84.21	15.61***	0.18	1.26
Control	54.60	45.13***	0.27	0.60

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%MNBn- frequency of binucleated cells containing micronuclei

Figure 4.25: MICRONUCLEUS INDUCTION BY TAUROCHOLIC ACID AT pH 5.5

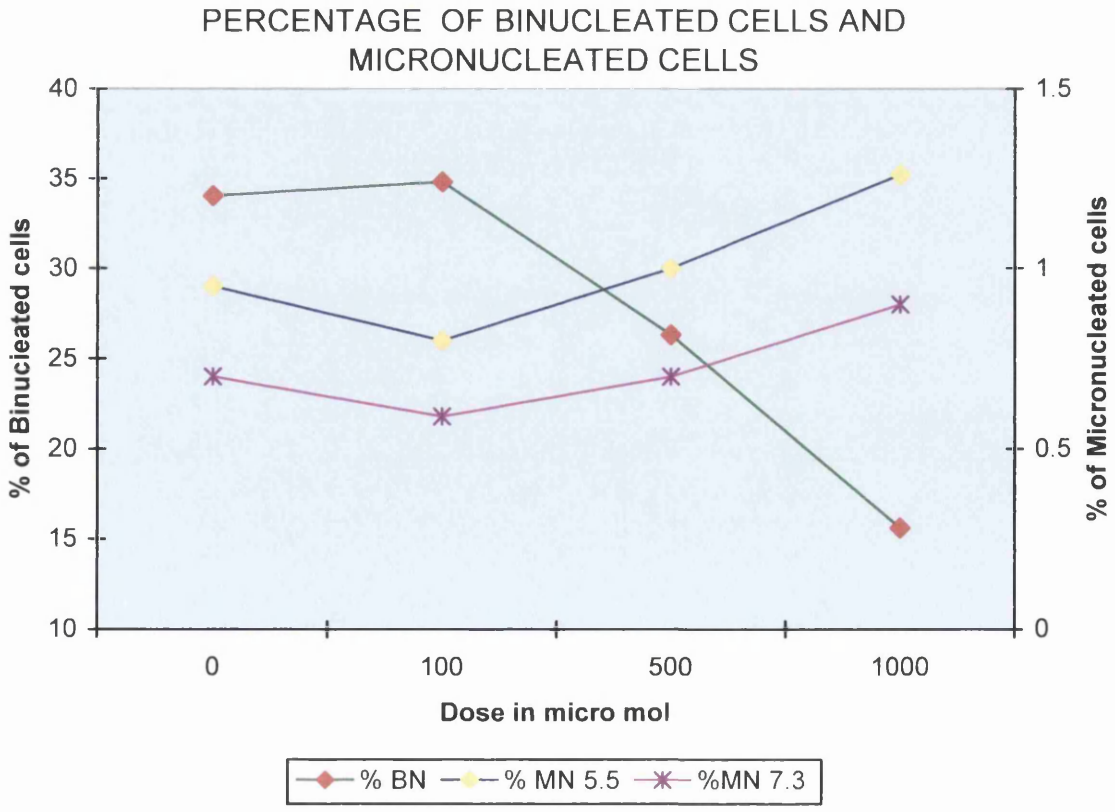


Table 4.23 Mean data for induction apoptosis and necrosis by Taurocholic acid at pH5.5

Dose in µmol/l	%Mono	%Binu	%Multi	%Apoptosis	%Necrosis
000	51.59	36.71	1.19	0.99	9.52
100	48.68	32.64	0.76	1.69	16.22**
500	55.66	13.52	0.99	1.59	28.23***
1000	46.73	16.24	0.99	2.38	33.66***

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%apoptosis- frequency of apoptotic cells
%necrosis- frequency of necrotic cells

Figure 4.26 Induction of apoptosis and necrosis by taurocholic acid at pH 5.5

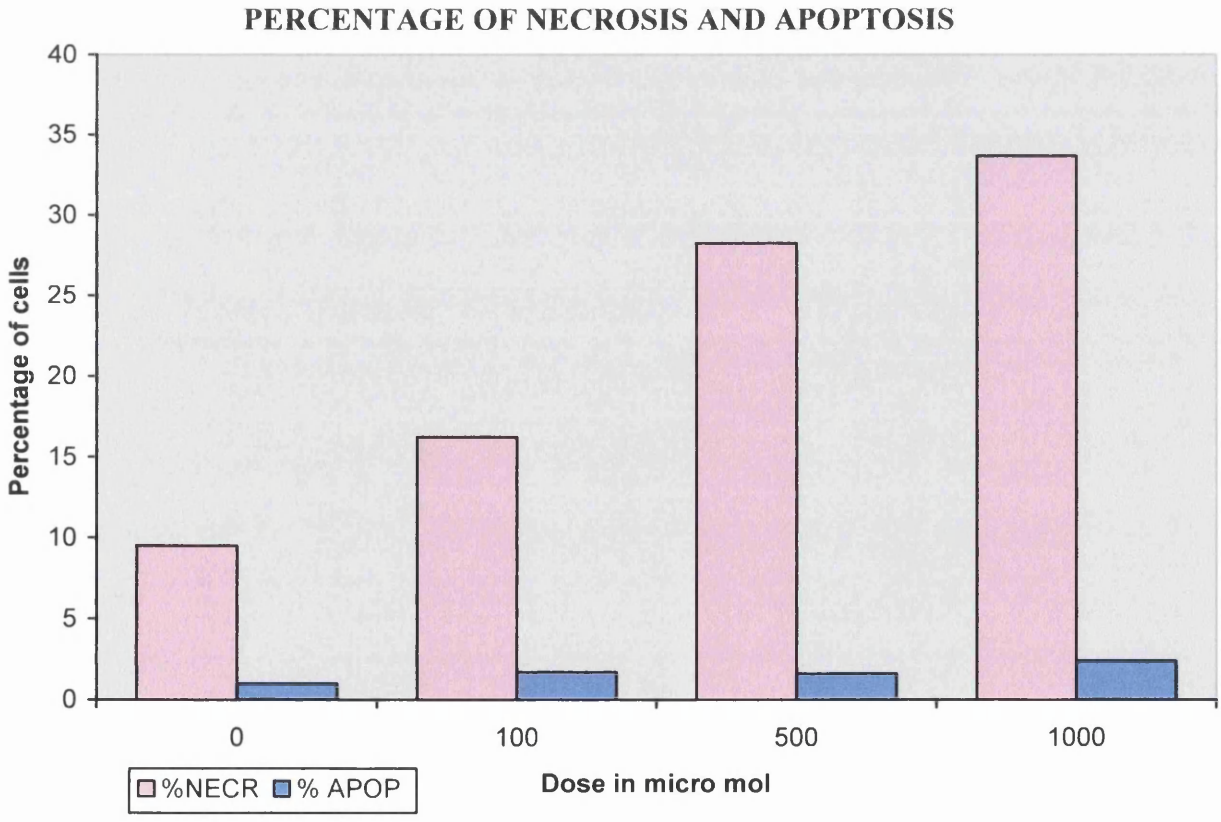


Table 4.24: Data for MN induction for Glycocholic acid treated OE33 cells at **pH 5.5**

Dose in µmol/l	%Mono	%Binuc	%Multinucleate	%MN BN
00	63.46	36.29	0.24	0.98
100	66.57	33.21***	0.22	0.72
500	74.24	25.51***	0.25	0.71
1000	85.22	14.58***	0.20	1.07
Control pH 7.4	51.98	47.73***	0.29	0.70

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%MNBn- frequency of binucleated cells containing micronuclei

Figure 4.27: MICRONUCLEUS INDUCTION BY GLYCOCHOLIC ACID AT pH 5.5

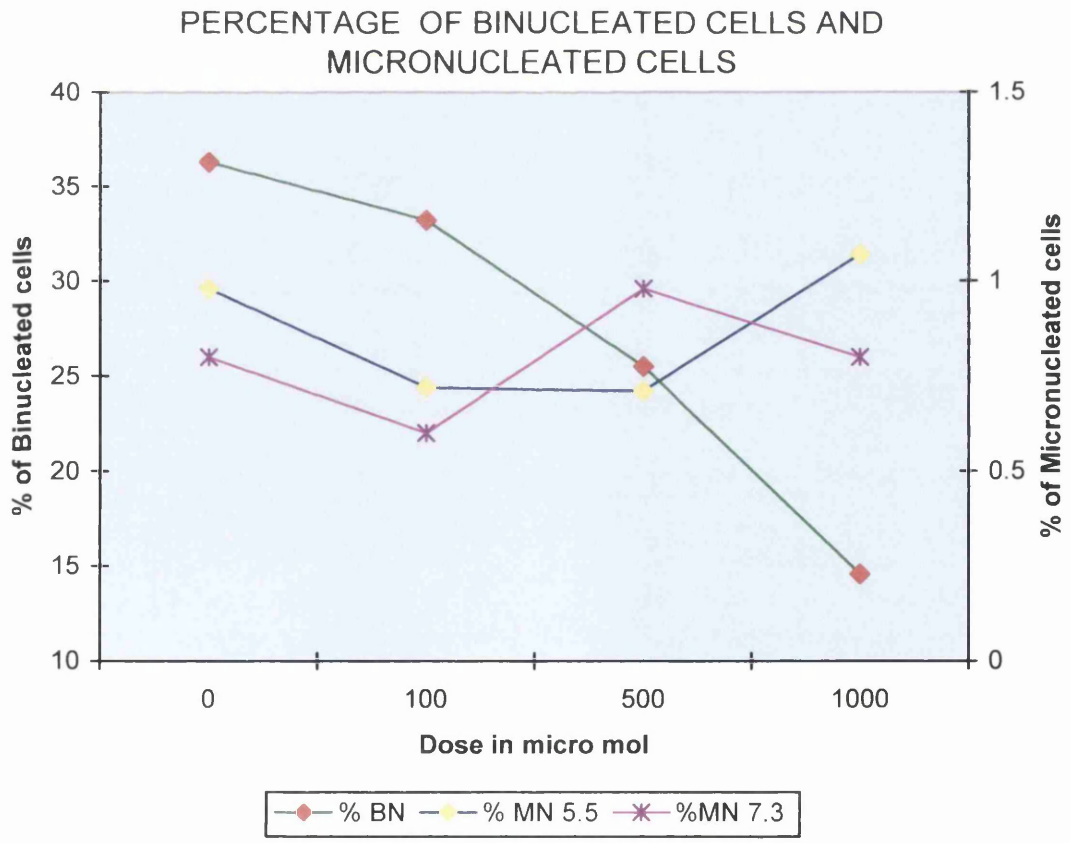


Table 4.25: Data for Induction apoptosis and necrosis by Glycocholic acid at **pH 5.5**

Dose in µmol/l	%Mono	%Binu	%Multi	%Apoptosis	%Necrosis
000	58.45	31.26	0.97	1.36	12.04
100	59.20	16.51	0.95	2.09	21.52***
500	52.02	14.26	0.77	1.54	31.41***
1000	55.42	10.85	0.79	1.76	31.16***

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%apoptosis- frequency of apoptotic cells
%necrosis- frequency of necrotic cells

Figure 4.28: Induction of apoptosis and necrosis by glycocholic acid at pH 5.5

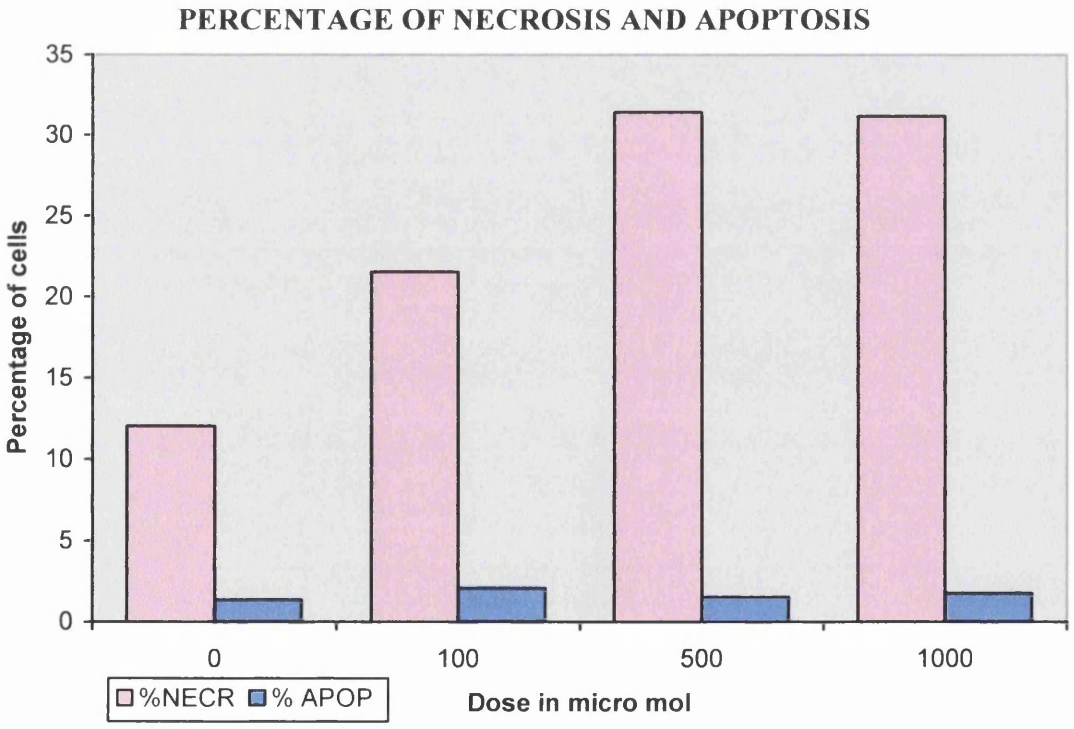


Figure 4.26: Data for MN induction for Taurodeoxycholic acid treated OE33 cells

Dose in µmol/l	%Mono	%Binuc	%Multinucleate	%MN BN
00	57.41	32.35	0.36	1.04
100	69.12	30.48	0.31	0.88
500	80.61	19.04***	0.32	1.35
1000	85.46	14.25***	0.29	1.15
NORMAL CONTROL	50.02	48.98***	0.50	0.81

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%MNBn- frequency of binucleated cells containing micronuclei

Figure 4.29: MICRONUCLEUS INDUCTION BY TAURODEOXYCHOLIC ACID AT pH 5.5

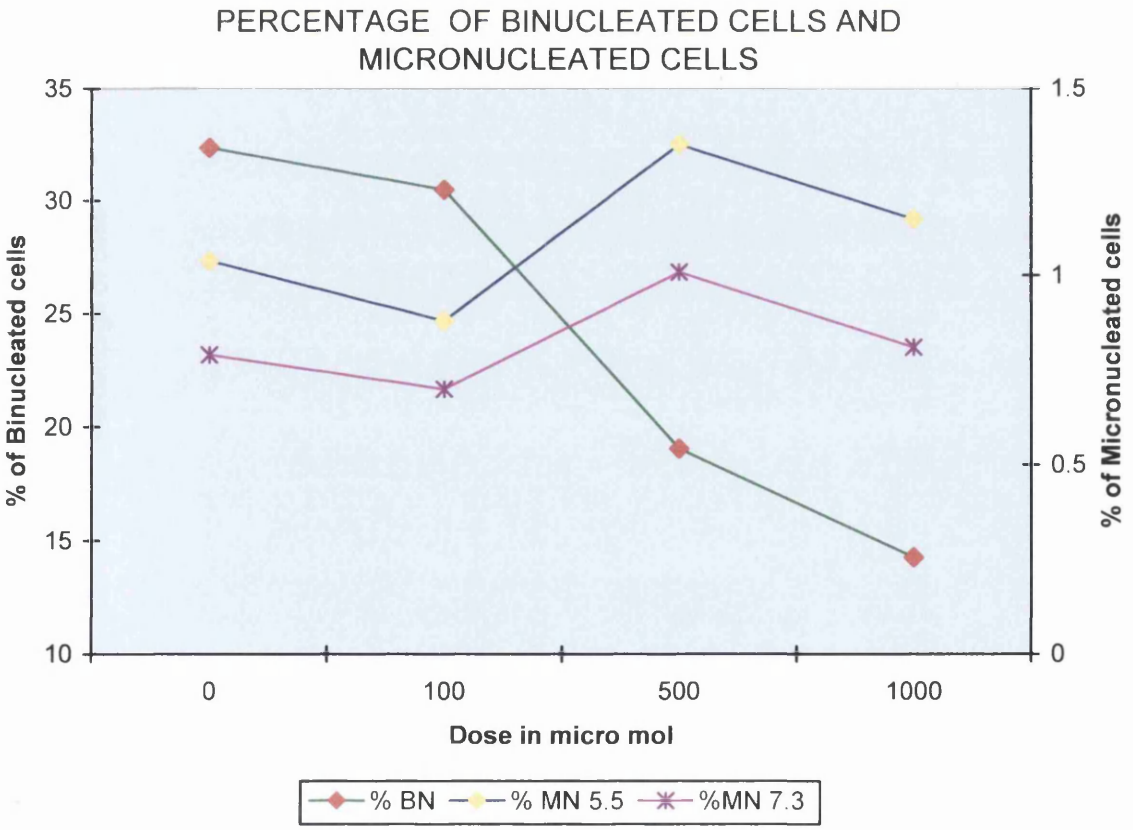


Table 4.27: Data for Induction Apoptosis and necrosis by Taurodeoxycholic acid

Dose in µmol/l	%Mono	%Binu	%Multi	%Apoptosis	%Necrosis
000	58.26	31.03	0.91	1.63	8.17
100	56.83	26.20	0.92	1.29	14.76***
500	41.96	18.43	0.78	1.57	37.25***
1000	49.61	18.90	0.98	1.18	29.53***

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%apoptosis- frequency of apoptotic cells
%necrosis- frequency of necrotic cells

Figure 4.30: Induction of apoptosis and necrosis by taurodeoxycholic acid

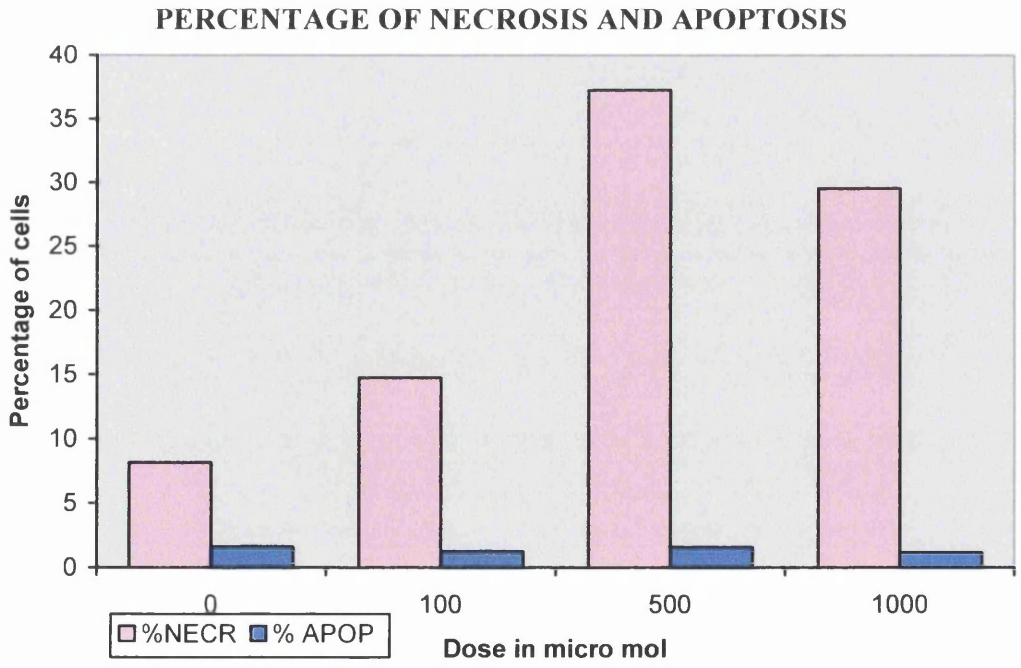


Table 4.28: Data for MN induction for Glycodeoxycholic acid treated OE33 cells

Dose in µmol/l	%Mono	%Binuc	%Multinucleate	%MN BN
00	67.68	32.06	0.26	1.05
100	72.07	27.68***	0.25	0.95
500	85.82	13.79***	0.38	1.47
1000	92.72	6.89***	0.30	1.67
NORMAL CONTROL	52.59	46.76***	0.65	0.89

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%MNBn- frequency of binucleated cells containing micronuclei

Figure 4.31: MICRONUCLEUS INDUCTION BY GLYCODEOXYCHOLIC ACID AT pH 5.5

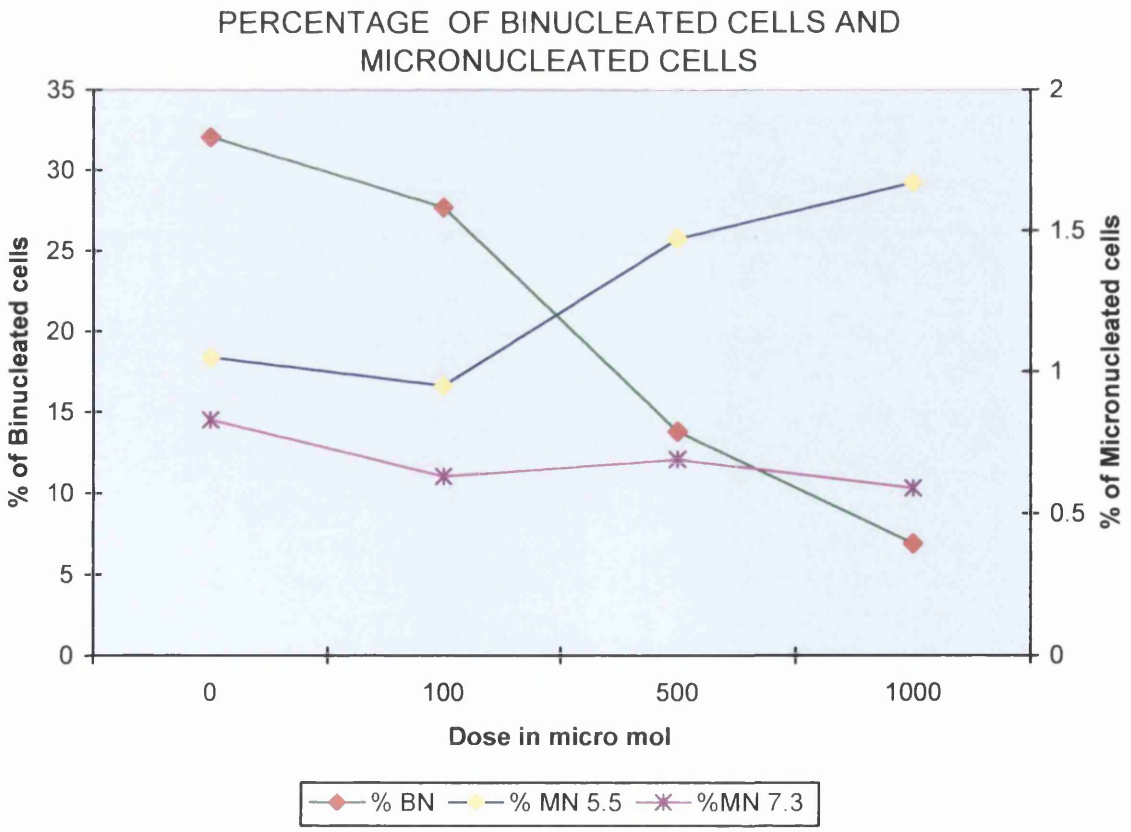


Table 4.29: Data for Induction Apoptosis and necrosis by Glycodeoxycholic acid

Dose in µmol/l	%Mono	%Binu	%Multi	%Apoptosis	%Necrosis
000	55.15	32.25	1.34	0.95	10.31
100	55.34	28.57	0.72	1.45	13.74
500	50.85	15.94	0.57	2.28	30.36***
1000	47.06	17.65	1.10	1.47	32.72***

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%apoptosis- frequency of apoptotic cells
%necrosis- frequency of necrotic cells

Figure 4.32: Induction of Apoptosis and Necrosis by Glycodeoxycholic Acid

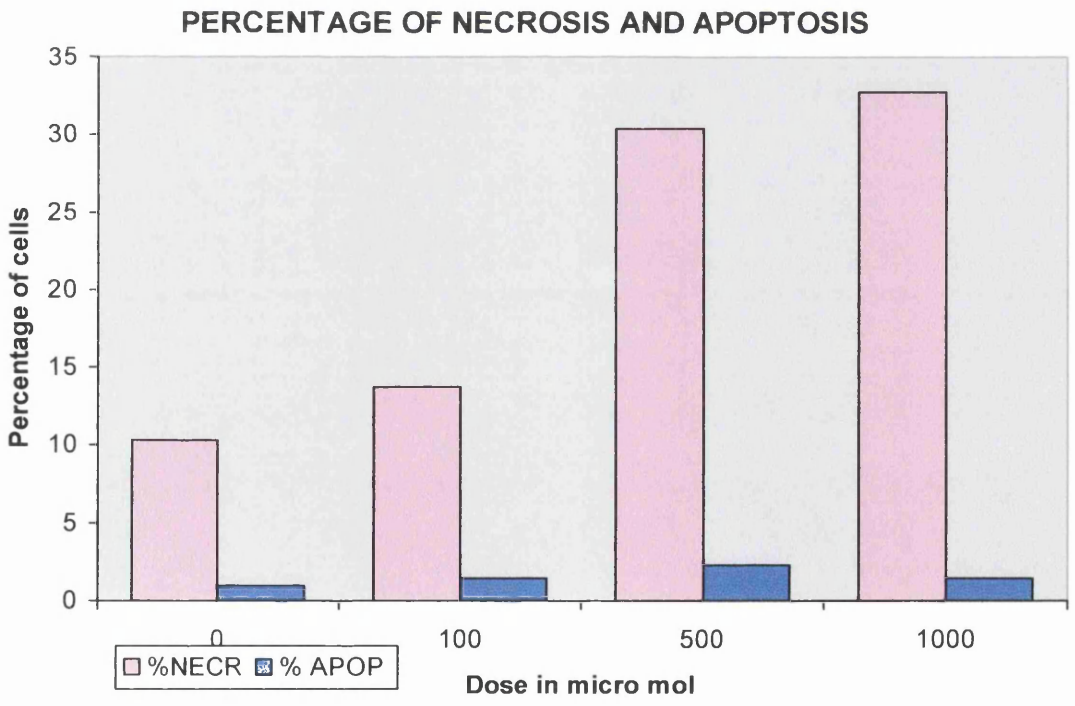


Table 4.30: Data for MN Induction for Deoxycholic Acid treated OE33 Cells

Dose in µmol/l	%Mono	%Binuc	%Multinucleate	%MN BN
00	62.96	36.82	0.30	0.96
50	78.044	21.70***	0.25	1.53
100	86.23	13.59***	0.173	2.68**
200	89.61	10.19***	0.19	2.88**

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%MNBn- frequency of binucleated cells containing micronuclei

Figure 4.33: MICRONUCLEUS INDUCTION BY DEOXYCHOLIC ACID AT pH 5.5

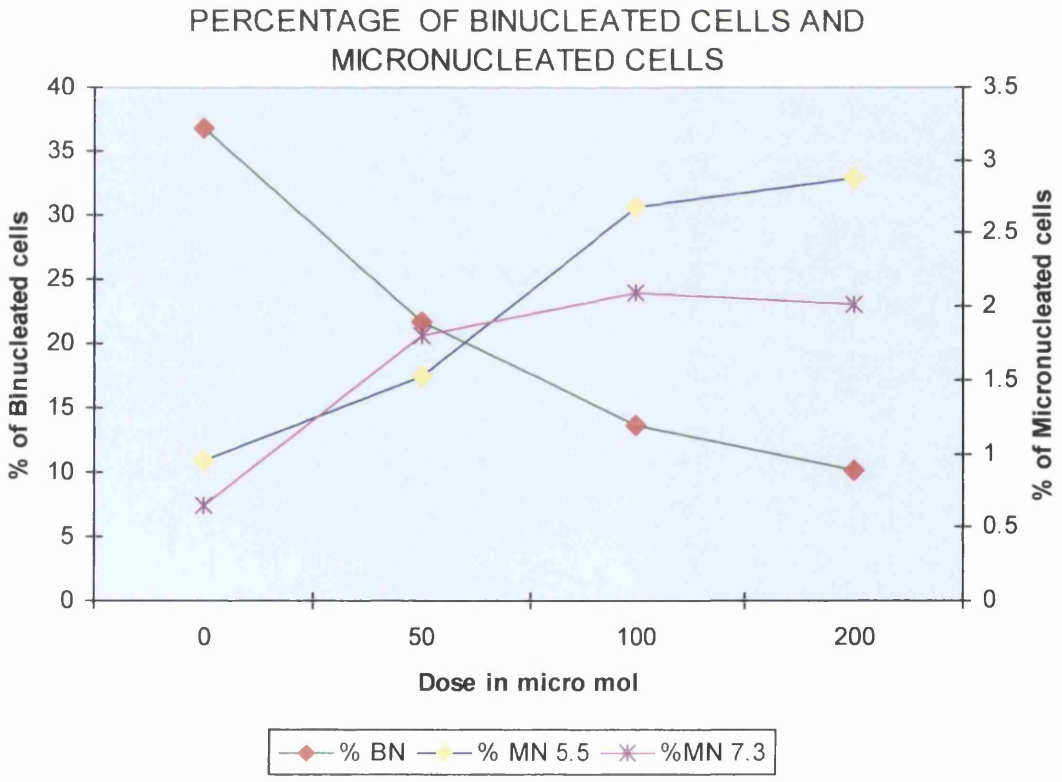


Table 4.31: Data for Induction Apoptosis and Necrosis by Deoxycholic Acid

Dose in µmol/l	%Mono	%Binu	%Multi	%Apoptosis	%Necrosis
000	46.85	42.52	1.18	1.18	8.27
50	46.66	29.71	1.14	2.29	20.19***
100	42.67	20.87	0.74	2.97*	32.84***
200	50.97	15.06	0.77	2.70	30.50***

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%apoptosis- frequency of apoptotic cells
%necrosis- frequency of necrotic cells

Figure 4.34: Induction Of Apoptosis And Necrosis By Deoxycholic Acid

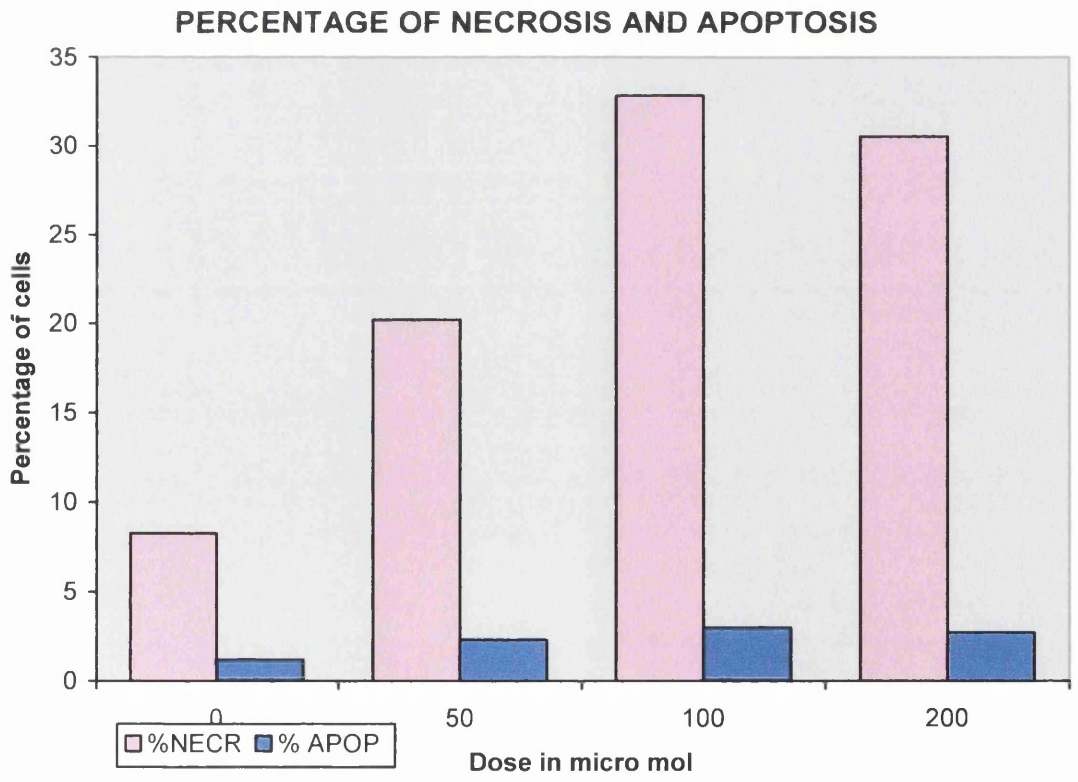


Table 4.32: Data for MN Induction for Cholic Acid treated OE33 Cells

Dose in µmol/l	%Mono	%Binuc	%Multinucleate	%MN BN
00	64.78	35.02	0.20	0.72
50	63.72	32.49***	0.19	0.76
100	75.15	24.7***	0.19	0.76
200	84.99	14.81***	0.20	1.09
500	90.94	08.90***	0.17	1.36

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%MNBn- frequency of binucleated cells containing micronuclei

Figure 4.35: MICRONUCLEUS INDUCTION BY CHOLIC ACIDAT pH 5.5

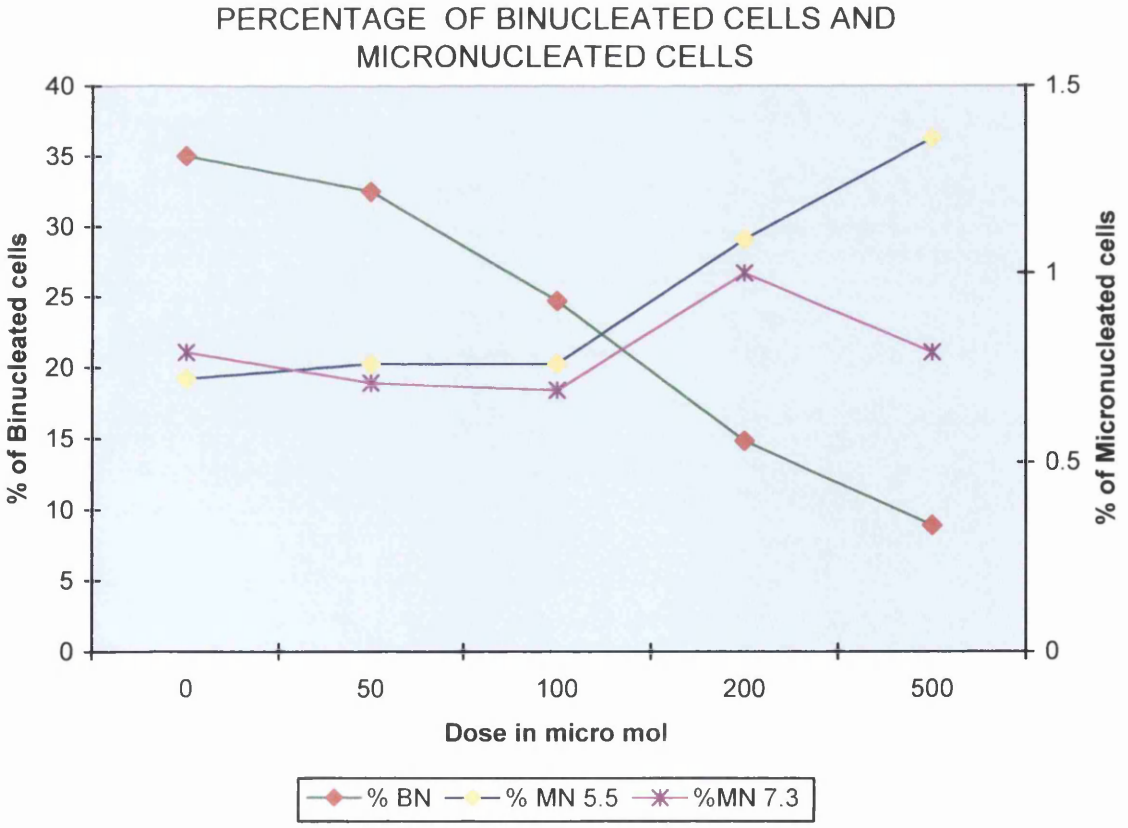
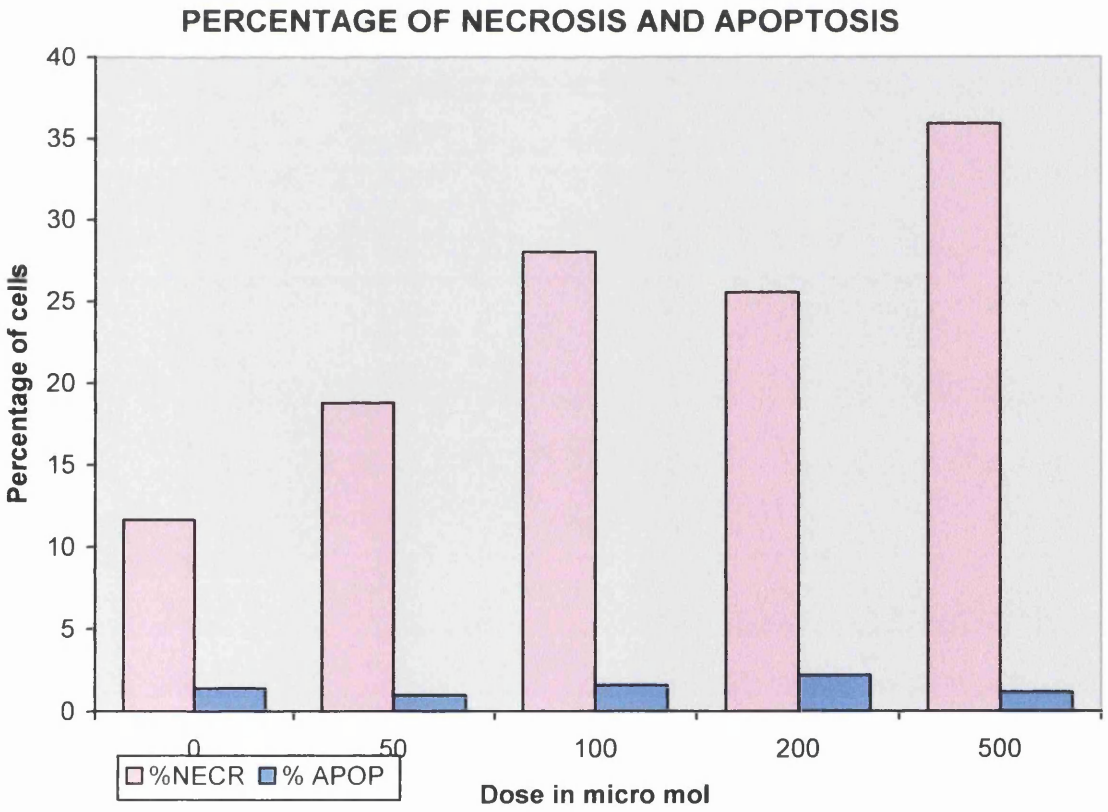


Table 4.33: Data for Induction Apoptosis and Necrosis by Cholic Acid

Dose in µmol/l	%Mono	%Binu	%Multi	%Apoptosis	%Necrosis
000	48.42	37.55	0.99	1.38	11.66
50	50.88	28.38	0.98	0.98	18.79**
100	40.27	28.79	1.36	1.56	28.02***
200	44.36	26.93	0.99	2.18	25.55***
500	43.38	18.62	0.96	1.15	35.89***

*P<0.05 **P<0.01 ***P<0.001
%Mono- frequency of mononucleated cells
%Binucleate- frequency of binucleated cells
%Multinucleate- frequency of cells containing more than 2 nuclei
%apoptosis- frequency of apoptotic cells
%necrosis- frequency of necrotic cells

Figure 4.36: Induction of Apoptosis and Necrosis by Cholic Acid



4.4 DISCUSSION

BARRETT'S OESOPHAGUS

There has been an increasing interest in Barrett's oesophagus in the last few years. This is because of its strong association with oesophageal adenocarcinoma, the incidence of which has been rapidly increasing in the last few decades. Surgical resection remains the standard treatment for early localised cancers. Despite improvements in surgical techniques, radiation and chemotherapy, the prognosis of this cancer remains poor (less than 25% of patients live for 5 years after oesophagectomy) (Ilson 2003; Wu *et al.,* 2003). The cause of the increasing incidence of this cancer is not known. A proper understanding of the aetiology and molecular events that characterize the transition to carcinoma is important for early detection and development of new preventive strategies to improve the prognosis for this grave disease. Although BE is a well recognised precursor of oesophageal adenocarcinoma, the aetiology, pathogenesis, prevalence and malignant risk of this lesion are still not clear. BE is an acquired condition which results from chronic DGER. Studies show that reflux of gastric and duodenal content are the most important factors in the development of this condition. Almost 10% of patients with GERD symptoms have BE (Spechler 2000).

Patients with BE have been shown to have increased reflux of acid and bile. BE possibly results from a complex interaction between acid exposure, bile, environmental factors and genetic susceptibility. More recently a number of studies have shown that bile acids may be one of the important factors involved in the pathogenesis of BE.

Several studies have evaluated the effects of bile acids (mostly DCA) on colonic cell lines, hepatocytes and pancreatic cancer cell lines and oesophageal cell lines.

No studies have been performed in the past to establish the genotoxic and cytotoxic potential of various bile acids on oesophageal cells in concentrations which are physiologically relevant to patients with BE. Knowledge about the genotoxic and cytotoxic potential of the various bile acids is limited. This is because most of the available data focus mostly on the effects of DCA on cell lines. The aim of our study was to fully evaluate the genotoxic and cytotoxic effects of the various bile acids on oesophageal cell lines. We therefore investigated the genotoxicity and cytotoxicity of the six different bile acids commonly encountered in patients with BE using oesophageal cell culture model. Six bile acids namely TCA, GCA, TDCA, GDCA, DCA and CA were used in the concentration range normally found in patients with BE and GERD. The maximum concentration was limited by the toxicity of the individual bile acids.

MN ASSAY

The cytokinesis-blocked micronucleus assay (CBMN) was used to evaluate the cytotoxic and genotoxic effects of the bile acids. The CKMB has been widely used as a sensitive screening test for the detection of chromosomal aberration and has emerged as one of the preferred methods for assessing chromosomal damage. Not only is it simpler and quicker than the traditional chromosome analysis it is also found to be equally effective. The combination of cytokinesis blocked Micronucleus assay and CREST (kinetochore) staining provides a rapid and simple means of analysing the induction of numerical chromosomal aberration. This technique can detect both

clastogenic agents which cause chromosomal breaks as well as aneugens which induce aneuploidy.

CYTOTOXICITY

All the bile acids were found to have cytotoxic and growth inhibitory effect as demonstrated by the decrease in proportion of the bi-nucleate cells. The decline in the frequency of bi-nucleated cells was accompanied with an accumulation of mono-nucleated cells.

The cytotoxic effect of DCA was greater than CA. The un-conjugated bile acids DCA and CA had a greater cytotoxic effect than the conjugated bile acids (TCA, GCA, TDCA and GDCA). All of the conjugated bile acids demonstrated a similar range of cytotoxicity. The cytotoxic effect of all the bile acids had a linear relationship to the dose. The cytotoxic effect of the bile acids (both the conjugated and un-conjugated bile acids) was found to be markedly increased in acidic pH.

Cytotoxicity of the bile acids in-vitro has been demonstrated by a number of studies (Salo JA *et al.,* 1984, Hopwood D *et al.,* 1981, Kivilaakso E *et al.,* 1980, Bachir *et al.,* 1982, Latta RK 1993). Bachir et al 1982 showed that conjugated bile acids in the range of 1-3mmol/l had a significant cytotoxic effect on oesophageal organ culture at neutral pH. Our findings are in agreement with this, and in addition we have shown that relatively small concentrations of bile acids (DCA 50 – 100μmol and conjugated bile acids >500μmol) can induce cytotoxic effects. This finding has an implication because even a relatively small quantity of bile reflux can cause damage in patients with BE. Furthermore, the cytotoxic effect of the bile acids was found to be dose dependent.

Kivilaakso E *et al.*, (1980) and Salo et al, (1980) found that the cell damage induced by bile acids was pH dependent. They found that conjugated BA (TCA) exerted their

cytotoxic effect at pH 3. 5 whilst un-conjugated BA (CA and DCA) exerted their cytotoxic effect at pH 7.4. Our findings do not agree with this, although it has to be noted that our experiments were conducted at a slightly higher pH (pH5.5 versus pH3.5). Our outcome was consistent with that of Hopwood et al (1981) who found that high and moderate concentration of all the common bile acids damaged the oesophagus irrespective of the pH. We found that lowering the pH of the medium increased the cytotoxic effect of all the tested bile acids in general. This shows that bile acids and low pH have a synergistic effect on inducing cell damage in oesophageal cells. Therefore bile acids in combination with acid can cause more damage compared to bile or acid alone.

GENOTOXICITY

The information regarding genotoxicity of various bile acids is limited. Most of the available data relates to the effects of DCA. In our study all the bile acids except DCA failed to induce statistically significant micronuclei. The degree of micronucleus induction remained constant irrespective of the dose and the degree of cytotoxicity. The induction of micronuclei with these bile acids was found to be slightly higher at acidic pH though this was not found to be statistically significant. There is no comparable data available in the literature about the genotoxicity of the conjugated bile acids.

Our results show the DCA induced statistically significant induction of micronuclei in the OE33 cells. The induction of micronucli was found to be at a maximum at doses between 100 and 200μmol. A further increase in the dose resulted in an increase in the percentage of MN although the number of bi-nucleate cells counted decreased profoundly due to increased cytotoxicity. Further evaluation of the mechanism of MN

induction by DCA using kinetochore staining showed that the micronuclei were predominantly kinetochore negative. This indicates that DCA has a predominantly clastogenic effect.

Unlike most other bile acids DCA has been extensively studied in the past. There is increasing evidence to show that DCA acts as a tumour promoter (Debruyne *et al.*, 2001) DCA has been shown to act as a tumour promoter by influencing cell signaling gene expression. DCA has been shown to increase cox-2 expression in oesophageal cells (Shirvani *et al.*, 2000, Zhang *et al.*, 1999). Studies have shown that the tumour promoting activity of DCA is closely related to activation of protein kinase C and AP-1 (Qiao *et al.,* 2000; Qiao *et al.,* 2002).

Despite extensive studies on the effects of DCA the information regarding its genotoxic activity is very limited and contradictory. Very few studies have demonstrated the DNA damaging effects of DCA. The earliest reports about DNA damaging effects of DCA was reported by Kandell *et al.,* and Cheah *et al .,* (Cheah *et al.,* 1990; Kandell *et al.,* 1991). They showed that bile acids, DCA in particular, could damage DNA in both bacterial and mammalian cells. More recently Venturi et al (1997), Powolny et al (2001) and Glinghammar *et al* (2002) have shown that DCA can induce DNA damage in colon cancer cells using comet assay. So far there are no studies which have demonstrated genotoxic effect of DCA on oesophageal cell lines. Our findings agree with the above studies. We found that concentrations of DCA similar to the ones observed in the above studies (100-300μmol) had a significant genotoxic effect. We also found that similar concentrations induced significant apoptosis of the OE33 cells. This data supports the findings of previous studies (LaRue *et al.,* 2000; Powell *et al.,* 2001; Powolny *et al.,* 2001; Milovic *et al.,* 2002).

As observed in previous studies the induction of apoptosis by DCA was at a maximum between 100 and 200μmol. At higher concentration DCA induced cell death was increasingly due to necrosis rather than apoptosis. The level of apoptosis remained constant with the other bile acids.

There is accumulating evidence that DCA can induce apoptosis. Apoptosis, or programmed cell death, is a mechanism that allows a cell to destroy itself when stimulated by an appropriate trigger (Wyllie *et al.,* 1980; Barr *et al.,* 1994; Schwartz *et al.,* 1995; Ellis *et al.,* 1996). This can be triggered by various stimuli, one of which is DNA damage secondary to exposure to genotoxic agents.

Mammalian cells respond to DNA-damaging agents by activating cell cycle checkpoints. This can lead to cell cycle arrest, apoptotic cell death, and transcriptional induction of genes involved in DNA repair. Arrest of cell cycle enables the cell tor repair the defective DNA. Repair of damaged DNA enables the cell to cycle to resume. On the other hand if the damage is too extensive, the cell will undergo apoptosis. Genotoxic DNA damaging agents may activate both membrane death receptors and the endogenous mitochondrial damage pathway leading to cell death via apoptosis.

Our studies showed that the proportion of apoptotic cells increased with the dose of DCA and was accompanied by induction of DNA breakage in the OE33 cells. We therefore assume that apoptosis in the OE33 was induced by DNA damage secondary to exposure to genotoxic concentrations of DCA. These studies indicate that the cytotoxic effects of DCA are partly mediated by the inhibition of DNA synthesis, induction of DNA damage and apoptosis.

On the other hand no significant apoptosis or DNA damage was observed with the other bile acids indicating that the cytotoxicity mediated by these bile acids was

possibly due to their direct destruction of cell membrane secondary to their detergent/lipid peroxidation effect. The experiments were repeated in acidic pH to mimic the normal in-vivo environment and to evaluate the influence of acid on the cytotoxicity and genotoxicity of the bile acids. In acidic medium (pH 5.5) the proportion of bi-nucleate cells was lower and the proportion of micro-nucleated bi-nucleate cells was higher than at neutral pH. There was also an increase in the proportion of necrotic cells at acidic pH. Although the induction of MN by the bile acids in the acidic pH was slightly higher than at neutral pH, it was not found to be statistically significant. DCA in particular showed a greater induction of MN at acidic pH. This has to be interpreted with caution as studies have shown low pH itself can be clastogenic to certain cell lines (Scott *et al.,* 1991; Morita *et al.,* 1992). The clastogenicity seen with low pH can vary with the type of cell lines and be associated with different degree of cytotoxicity. It has been shown that clastogenicity evaluated at low pH can be unreliable when the degree of cytotoxicity is greater than 50%. The overall data indicates that the bile acids are cytototoxic and that DCA exerts significant genotoxicity on oesophageal cell lines at relatively low concentrations. These findings are important because it is well documented that chromosome damage is strongly and positively associated with cancer risk (Mitelman *et al.,* 1990).

The in-vitro effect of DCA on the cell toxicity and DNA damage requires further detailed investigation. These findings, however, provide further evidence for the involvement of bile acids in the malignant transformation of BE and development of oesophageal adenocarcinoma. We therefore conclude that bile acids may play a significant in the pathogenesis of BE and its progression to adenocarcinoma.

CHAPTER FIVE

GENERAL DISCUSSION

Our work has focused on the various aspects of Barrett's oesophagus. The main objective of this study was to use molecular and cellular approaches to understand the mechanism of the development of Barrett's oesophagus and the progression of BE to OADC. Understanding these mechanisms will ultimately help in designing effective strategies for the management and treatment of Barrett's Oesophagus. Identification of a biomarker which could predict the progression of BE to OADC would help in the early detection and management of this dreadful condition which has a very poor prognosis. The general discussion highlights the findings described in the previous chapters of this thesis.

5.1. DEVELOPMENT OF AN IN-VITRO MODEL

One of the aims of this work was to develop an in-vitro model of cell culture to study the molecular aspects of BE (**chapter two**). In-vitro models play an invaluable role in understanding tumour biology providing some very important insights into the genesis of cancer. They also provide a well defined environment for cancer research in contrast to the complex host environment of an in-vivo model. Primary Barrett's cell culture would serve as an ideal model to study the transition of BE to OADC. Such a model would not only enable us to study the mechanism involved in neoplastic progression but would also allow us to detect biomarkers which could reliably predict the progression of BE to OADC. Very few studies have been performed in the past in developing a cell culture model from the pre-malignant Barrett's cells. Most studies have had very limited success with few having used this technique subsequently for cytogenetic studies. Despite successful attempts to obtain long-term culture by some workers the widespread use of this method for cytogenetic analysis has been limited.

This is because of several reasons including lack of easy reproducibility, infection and overgrowth of fibroblasts and limited life span of these cells. Unlike cancer cells, BE cells lack clonogenicity and therefore have finite life span. To date none of the workers have been able to achieve immortal cell lines from BE cells. We were able to achieve long term culture although the success rate was very poor. Although these cells survived more than 8 weeks it was extremely difficult to subculture them or obtain metaphase spreads. In our opinion use of long term culture for FISH studies is difficult and labour intensive. It offers no unique advantage over other quick and easy methods such as brush cytology or short term culture. At the beginning of the study we aimed to obtain cell lines from patients with BE in the hope of evaluating the early cytogenetic changes and obtaining a long term cell cultures from BE cells. Although a few studies have described success with long term culture of BE, continuous oesophageal cell lines are yet to be obtained. Despite the failure to obtain continuous cell lines we managed to obtain and optimise the technique for short term culture of BE cells. This technique was found to be relatively easier, reproducible and had the unique advantage of being more accurate in representing the changes seen in the tissue of origin.

5.2 FISH STUDIES

Our study also aimed at analysing in detail some of the changes that were previously analysed in our laboratory using CGH. We had previously found that amplification of chromosome four and eight was seen in patients with HGD and OADC (Croft *et al.*, 2002). The aim of our study was to see if these changes were seen in early stages of BE. The latter part of **chapter two,** deals with interphase FISH analysis of Barrett's cells. Although we were successful in analysing the changes with FISH, our work was limited owing to difficulty in obtaining cells for the studies. During the initial part of

the study most of the cells were lost (senescence or infection) owing to our attempts to obtain long term culture. All the samples for FISH analysis were obtained from short term culture which we were able to optimize towards the end of the study.

5.21 CHROMOSOME FOUR HYPERPLOIDY

Interphase FISH was performed on cultured cells using CEP probes to look for aneuploidy of chromosome four and eight. Cell samples for interphase FISH can be obtained by several means including cell culture, touch imprint technique, brush cytology or even by using paraffin sections. Touch imprint technique has been used in the analysis of solid tumours (prostate, kidney) and has been found to be a relatively simple, rapid, and reliable method for detecting chromosome abnormalities. Data is however lacking for use of this technique in mucosal lesions like BE, although studies from our own laboratory show that it is fairly reliable (Croft *et al.*, 2002). Use of paraffin fixed material is possible although it is more difficult to process and interpret the results than the conventional methods. Brush cytology on the other hand is very easy, quick and reliable compared to cell culture, although short term culture has the advantage of providing increased number of cells for cytogenetic studies.

Our previous observation of hyperploidy of chromosome four and eight were based on CGH studies which can detect changes when present in large proportion of cells. The aim of our study was to see if these changes were seen in relatively early stages of BE. We used interphase FISH technique, which is sensitive and can detect changes found in smaller proportion of the cells. We found that amplification of chromosome four and eight was common in the early stages of BE. Owing to the small number of samples analysed it would be difficult to make sensible conclusions from these results alone. However, these findings have been confirmed in larger numbers of patients by

using interphase FISH and brush cytology by Doak *et al* (2003). We can therefore conclude that interphase FISH is a reliable, accurate and sensitive technique for detecting numerical chromosomal abnormalities in patients with BE. We have shown that these alterations occur early during the neoplastic transformation of Barrett's mucosa. These regions may harbour candidate genes involved in the pathogenesis of this malignancy.

5.3 CHARACTERISATION OF OE33 CELL LINES

Chapter 3 deals with the characterization of OE33 cells. The genomic re-arrangements of only a few oesophageal cell lines have been completely characterized. The aim of this study was to identify chromosome regions that may contain genes important for the development of oesophageal carcinomas and to identify genetic markers associated with tumour progression. We analyzed the OE33 cells using a combination of G-banding and FISH techniques. The G-banding analysis revealed a very complex karyotype with gross polyploidy and presence of additional material of unknown origin. Owing to the complex karyotypic arrangements seen with G-banding, we used chromosome-specific probes to better characterize the abnormalities. The use of these FISH-based screening techniques allowed us to detect several complex chromosomal changes. Uncertainties seen with G-banding were removed only after hybridization with chromosome-specific probes. The combined analysis revealed a near tetraploid karyotype with almost all the chromosomes demonstrating structural rearrangements. Our study confirms the high frequency of chromosomal structural and numerical aberrations in the OE33 oesophageal cell lines. We provide a detailed description of chromosomal translocations in the OE33 cells

although they are complex and difficult to interpret. Some of these changes may be useful to direct future intensive investigation of these chromosomal regions.

The findings of G-banding and FISH confirm the chromosomal instability of OE33 cells. Chromosomal instability is a hall mark of cancer cells the mechanism of which is poorly understood. The centrosomes which are involved in segregation of chromosomes play an important role in the development of aneuploidy. Centrosome and microtubule analysis was performed using immunofluorescence labelling technique. Our studies showed that there were striking defects in centrosomes number and size in the OE33 cells. The centrosomal abnormalities correlated well with chromosomal instability. Presence of centrosome imbalance and aneuploidy together suggests that mitotic error and faulty segregation could have resulted in the chromosomal instability. It has been suggested that centrosome amplification could be an early feature in neoplastic progression which could be followed by clonal selection of viable progenitor cells (Brinkley *et al.,* 1998). It would be useful to see if these changes are seen in early stages of BE.

5.4 ROLE OF BILE

Until today, the aetiology and pathogenesis of Barrett's oesophagus is poorly understood. Damage of the epithelial cells by various noxious agents seems to be the initial process in the pathogenesis of Barrett's oesophagus. Reflux of duodenal contents plays an important role in the genesis of BE and its progression to BE. The role of acid reflux in the genesis oesophagitis has been well recognised and was first elaborated by Winkelstein (Winkelstien 1935). Despite the fact that acid plays an important role in the pathogenesis of BE there seems to be factors other than acid which play an equally important role. This is based on the observation that BE and

OADC have been seen in patients following total gastrectomy. Oesophagitis has been noticed in patients following subtotal gastrectomy. Regression of oesophagitis has been seen in these patients following surgery for duodenal diversion. From studies in experimental models it is clear that bile acids alone can induce BE and oesophageal adenocarcinoma in rats. Several studies have shown that bile reflux is common in patients with BE. The composition of bile in these patients has been evaluated by several workers, and was found to be composed of both conjugated and un-conjugated BA. Studies have also shown that 10-25% of the patients with erosive oesophagitis have normal pH studies (Masclee *et al.,* 1990). 25-35% of the patients on recommended dose of PPI develop recurrent or progressive disease. All this shows that duodenal reflux does play a role in the genesis of BE and OADC.

5.41 CYTOTOXICITY AND GENOTOXICITY OF BILE ACIDS

We hypothesized that bile acids play a role in the damage of epithelial cells and are possibly involved in inducing DNA damage. While it is generally accepted that bile acids can damage cells the ability of different bile acids (particulary TCA, GCA, GDCA, TDCA, CA and DCA) to damage oesophageal cells has not been demonstrated in the past.

The *in vitro* MN assay was used to assess the cytotoxic and genotoxic potential of the bile acids on oesophageal cell lines. The *in vitro* MN assay was found to be an easy and reliable method to evaluate the mutagenic potential of the various bile acids. The MN assay has been well validated and the results have been found to be comparable to metaphase analysis. The CBMN assay is in fact a multi endpoint assay which simultaneously detects genotoxicity and cytoxicity, chromosmal breakage, chromosome loss, cell division inhibition, necrosis and apoptosis (Kirch-Volders *et*

al., 1997, Fenech, 2000). Use of CREST analysis further enables to distinguish between clastogens and aneugens.

Bile salts are essential components required for the absorption of fat. However their detergent property makes them potential cytotoxic agents. In **chapter 4**, we have examined the potential ability of the various bile acids to induce cell damage and DNA damage in oesophageal cell lines. Patho-physiologically relevant concentrations of the bile salts were found to induce dose-dependent cell toxicity and necrosis. Of all the bile acids DCA was found to be the most potent cytotoxic agents. The un-conjugated bile acids were found to be more potent than the conjugated bile acids in inducing cell damage. DCA and CA induced cytotoxicity at concentrations as low as 100μmol. All the conjugated bile acids had a similar range of cytotoxicity.

Our study has shown that relatively low concentrations of BA can induce cell damage. All the bile acids remained in solution both at pH 5.5 and 7.4. The cytotoxicity of all the bile acids was found to be significantly higher at pH 5.5 compared to pH 7.45.

Different studies in the past have demonstrated that DCA interacts directly at the DNA level resulting in disruption of genomic function. Studies have shown that DCA can induce DNA strand breaks in cells lines.and can lead to the formation of DNA adducts. There is evidence to show that DCA may act as a tumour promoter. Our study showed that all bile acids (except DCA) failed to induce micronuclei and hence lacked any significant genotoxicity in vitro. DCA on the other hand induced statistically significant micronuclei in the OE33 cells. The induction of micronuclei by DCA was at a maximum at doses between 100 and 200μmol. Kinetochore staining revealed that DCA was predominantly clastogen in nature. DCA induced apoptosis in dose dependent manner in the OE33 cells. DCA induced apoptosis was possibly

secondary to DNA damage. These findings may have important implications in the pathogenesis and management of BA.

5.5 FINAL CONCLUSION

The work described in this thesis has contributed to a better understanding of the several factors relating to in-vitro models of BE and the effects of commonly seen bile acids on oesophageal cell lines. The increase in incidence of BE together with the poor prognosis of OADC makes this condition extremely important. This increase, together with the dismal 5-year survival of patients with this cancer, has focused attention on its risk factors.

The initial aim of this study was to understand the early genetic changes in BE which could serve as a biomarker in identifying patients who could progress to OADC. This aim was to be achieved by obtaining short or long term in-vitro cell culture model of Barrett's. The entire success of the project was based on obtaining an in vitro-model of BE. We were partly successful in obtaining short term cultures although it was extremely difficult and labour intensive with inconsistent results and poor reproducibility. Our studies have also shown that long term cultures are difficult to obtain due to lack of clonogenicity of Barrett's cells. With limited success that was obtained from short term cultures we were able to perform cytogenetic studies on a small number of samples. Owing to an extreamly small number of samples studied it would be over-ambitious to draw a definite conclusion, although the findings were consitent with observations made by Doak *et al* (2003). Our two initial goals, namely obtaining an in vitro model and studying the early cytogenetic changes, were therefore not fulfilled. The study however shows that in the presence of adequate cells it is possible to perform interphase cytogenetic studies.

The subsequent aim of this study was to evaluate the role of bile acids in the pathogenesis of BE. Owing to inconsistency and difficulty in obtaining an in-vitro model from BE an alternate but stable and closely representative model was used (Barrett's adenocarcinoma cells- OE33 cells). The in vitro micronucleus assay a multi-end point assay, was used to evaluate the cytotoxicity and genotoxicity of bile acids. This study has clearly shown that both conjugated and un-conjuagated bile acids are cytotoxic at relativily low concentrations. However it is clear from our study that most of the bile acids lack genotoxic effect except for DCA. The study has answered our initial questions, and does suggest that DCA is the only candidate which would need further evaluation regarding its genotoxicity and carcinogenicity.

A number of conclusions can be drawn from our work the most important which have been enumerated below:

- Both short and long term cultures of BE difficult to obtain, labour intensive and is not easily reproducible. Although it is possible to obtain cells from short term cultures it is not a reliable technique to obtain material for cytogenetic studies. Not only are the results inconsistent but are also diffuclt to reproduce.
- Interphase FISH can be reliably used in the cultured cells to detect numerical chromosomal aberration.
- Chromosomal aberrations and centrosome abnormality are common in oesophageal adenocarcinoma cells, and that centrosome abnormalities correlates with the degree of aneuploidy.
- The cytokinesis blocked micronucleus assay has been successfully applied to evaluate the genotoxic and cytotoxic potential of the bile acids.

- Our results derived from in-vitro MN assay indicate that bile acids are cytotoxic at relatively low concentrations which are relevant to patients with BE. Un-conjugated bile acids were found to be more cytotoxic than conjugated bile acids. In general the cytotoxicity of the bile acids was exaggerated by lowering the pH of the medium.
- Our study demonstrates that DCA does exert significant genotoxic effect in oesophageal cell lines at concentrations as low as 100-200μmol/l. CREST anaylsis showed that DCA was clastogenic in action. The DNA damaging effect of DCA therefore needs further evaluation.
- None of the other bile acids (TCA, GCA, TDCA, GDCA and CA) produced any genotoxic effects *in vitro*.

Together the above findings support the hypothesis that bile acids can cause damage to the oesophageal epithelial cells in vitro.

5.6 CLINICAL IMPLICATIONS OF BILE REFLUX

Most authors believe that suppression of acid with PPI s can control bile reflux by reducing the volume gastric fluid. Studies have shown that treatment with adequate doses of PPI fails to suppress acid and bile reflux completely ((Marshall *et al.,* 1998; Manifold *et al.,* 2000; Sarela *et al.,* 2004). Suppression of acid with a Proton pump inhibitor in turn results in a high prevalence of gastric bacterial overgrowth. The presence of bacterial overgrowth markedly increases the concentration of unconjugated bile acids (Theisen *et al.,* 2000). This could lead to continued mucosal injury despite suppression of acid reflux. Hence prevention of both acid and bile reflux is important in patients with BE. Studies have shown that standard anti-reflux procedures are effective in preventing acid and bile reflux (Stein *et a*l., 1998). More

recently Gurski *et al* showed that more than one-third of patients with visible segments of Barrett's oesophagus undergo histologic regression after anti-reflux surgery (Gurski *et al.,* 2003). Csendes *et al* (2002) showed that the rate of failure of the standard anti-reflux procedure (Nissen fundoplication or Hill's posterior gastropexy) was higher in the patients with BE than in those with reflux oesophagitis owing to the persistence of duodenal reflux into the oesophagus (Csendes *et al.,* 2002). They treated their patients with aggressive surgery to prevent both acid and bile reflux. They performed anti-reflux surgery plus either a duodenal switch procedure or a partial distal gastrectomy with Roux-en-Y gastrojejunal anastomosis in patients with BE (Csendes *et al.,* 2002). They found that following surgery there was a significant decrease in acid reflux, and that duodenal reflux was completely abolished in almost all patients. None of these patients progressed to high-grade dysplasia or adenocarcinoma. It is therefore important to monitor bile reflux in patients with BE. In patients with advanced and persistent bile reflux, surgical options should be considered as treatment with PPI alone is insufficient to prevent bile reflux.

5.7 FUTURE PERSPECTIVES

The results of this research strongly support the role of bile acids in oesophageal injury and transformation of BE to oesophageal adenocarcinoma. Although the data suggests the possible role of BA in the pathogenesis of BE and OADC more studies are needed to confirm these findings. Our studies were conducted using commercial preparations of bile acids which although is not ideal, gives a good representation of the natural situation. A better model would be to use gastric aspirates from patient with GERD and BE. Studies in the past have shown that bile acids can be precipitated at low pH rendering them harmless at low pH. Use of natural bile acids from the aspirate obtained from patients can allow the naturally occurring interaction between

alkaline and acid components. This could also provide insight into the presence of any other genotoxic agents which could be of importance.

There are not many studies which have measured the exact composition and concentrations of bile acids in patients with GERD and BE. The currently utilised Bilitec technique, although sensitive in detecting presence of bile reflux is semi-quantitative at best. Hence more studies are required to study the exact composition.

Characterisation of the oesophageal cancer cells did show that centrosome defects were common and that they correlated well with aneuploidy. It will be interesting to see if centrosomal defects are seen in patients with early BE. Although our studies did not show any association between BA and induction of aneuploidy it would be worth investigating if naturally occurring duodenal contents can induce aneuploidy and centrosomal defects.

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APPENDICES

Appendix A: Outcome Of Barrett's Cell Culture.

Table A2.1 Showing the details of the outcome of Barrett's cell culture

PATIENT CODE	AGE	MALE	FEMALE	LGD	HGD	BE	SSBE	LSBE	EXP-GROWTH	EXP-NO GROWTH	EXPL-INFECTION	ENZY-GROWTH	ENZY-NO GROWTH	ENZY-INFECTION
101	54	1		1				1		1			1	
102	75		1		1			1			1			1
103	58	1		1				1			1			1
104	86		1			1	1			1			1	
105	55	1		1			1			1				1
106	37	1				1	1		1					
107	49	1			1			1			1			
108	61	1				1	1		1					
109	44	1				1	1			1				
110	55	1		1				1	1					
111	81	1			1			1			1			
112	75	1			1		1		1					
113	74	1		1			1			1				
114	66	1		1			1				1			
115	88	1			1			1			1			
116	73	1				1	1			1				
117	56	1				1	1		1					
118	49	1				1	1				1			
119	73	1				1	1		1					
120	79	1		1				1		1				
121	81	1			1			1			1			
122	54	1				1	1		1					
123	40		1			1	1		1					
124	54	1				1	1		1					
125	67	1				1	1		1					
126	81		1	1				1		1				
127	40	1				1		1			1			
128	66	1				1	1				1			
129	88		1			1	1			1				
130	87		1			1	1				1			
131	71	1		1			1				1			
132	61	1				1		1			1			
133	70		1			1	1			1				
134	52	1				1	1				1			
135	50	1		1			1		1					
136	38	1				1	1		1					
137	78		1	1			1			1				

138	62	1		1			1		1					
	63.89	30	8	12	6	20	26	12	13	11	14	0	2	3

Appendix B: Raw data from MN analysis at pH 7.4

TAUROCHOLIC ACID AT NEUTRAL PH The in vitro Micronucleus Assay														
Dose of Bile Acid	Cell counts						**% BN Cells**	**Mn in Binucleates**			**Mn in Mononucleates**			**%MicN BN**
	MN	**BN**	**3**	**4**	**4+**	**Total**		**1**	**2**	**3**	**1**	**2**	**3**	
00µmol	650	502	5	1	2	1160		4	0	0	3	0	0	
	564	499	7	3	3	1076		3	0	0	2	0	0	
Total	12 14	1001	12	4	5	2236	44.80	7	0	0	5	0	0	0.70
100µmol	716	504	5	6	1	1232		2	0	0	4	0	0	
	800	508	3	3	2	1316		4	0	0	3	0	0	
Total	1516	1012	8	9	3	2548	39.70	6	0	0	7	0	0	0.59
200µmol	794	503	1	4	1	1303		5	1	0	3	0	0	
	837	504	5	3	3	1352		5	0	0	2	0	0	
Total	1631	1007	4	7	3	2652	38.00	9	1	0	5	0	0	0.99
300µmol	1000	575	5	3	3	686		5	1	0	4	0	0	
	1084	452	3	1	2	1542		6	0		2			
Total	2084	1027	9	4	5	3129	32.80	11	1	0	6	0	0	1.16
400µmol	1100	500	5	2	4	1611		3	0	0	2	0	0	
	1109	516	3	2	2	1632		5			1			
Total	2209	1016	8	4	6	3243	31.33	8	0	0	3	0	0	0.79
500µmol	912	490	3	3	3	1411		3	0	0	2	0	0	
	952	510	5	2	3	1472		4			0			
Total	1864	1000	8	5	6	2883	34.70	7	0	0	2	0	0	0.70
600µmol	1200	493	3	4	4	1704		3	0	0	1	1	0	
	1252	510	1	2	3	1768		2			1	0		
Total	2452	1003	4	6	7	3472	28.90	5	0	0	2	1	0	0.50
700µmol	1020	501	4	3	5	1533		5	0	0	3	0	0	
	1096	505	3	2	4	1610		3			1			
Total	2116	1006	7	5	9	3143	32.00	8	0	0	4	0	0	0.79

TAUROCHOLIC ACID AT NEUTRAL PH The in vitro Micronucleus Assay														
Dose of Bile Acid	Cell counts						**% BN Cells**	**Mn in Binucleates**			**Mn in Mononucleates**			**%MicN BN**
	MN	**BN**	**3**	**4**	**4+**	**Total**		**1**	**2**	**3**	**1**	**2**	**3**	
800µmol	1350	552	5	2	3	1912		3	0	0	2	0	0	
	1361	560	3	1	4	1929		2	0	0	1	0	0	
Total	2711	1112	8	3	7	3841	28.95	5	0	0	3	0	0	0.45
900µmol	1248	500	4	5	3	1760		3	0	0	3	0	0	
	1255	502	2	4	2	1765		3	0	0	2	0	0	
Total	2503	1002	6	9	5	3525	28.43	6	0	0	5	0	0	0.60
1000µmol	1633	500	5	6	4	2148		5	0	0	2	0	0	
	1694	498	7	3	2	2204		4	0	0	2	0	0	
Total	3327	998	12	9	6	4352	23.00	9	0	0	4	0	0	0.90
2000µmol	1532	488	3	3	5	2031		3	0	0	3	0	0	
	1588	470	5	2	2	2067		4	0	0	2	0	0	
Total	3120	956	8	5	7	4096	23.33	7	0	0	5	0	0	0.73
3000µmol	1600	420	6	4	4	2034		4	0	0	1	0	0	
	1651	440	5	3	5	2104		2	0	0	1	0	0	
Total	3251	860	11	7	9	4138	20.80	6	0	0	2	0	0	0.70

GLYCOCHOLIC ACID AT NEUTRAL PH The in vitro Micronucleus Assay														
Dose of Bile Acid	Cell counts						**% BN Cells**	**Mn in Binucleates**			**Mn in Mononucleates**			**%MicN BN**
	MN	**BN**	**3**	**4**	**4+**	**Total**		**1**	**2**	**3**	**1**	**2**	**3**	
00µmol	502	503	3	3	5	1016		4	0	0	2	0	0	
	487	506	4	2	3	1002		4	0	0	2	0	0	
Total	989	1009	7	5	8	2018	50.00	8	0	0	4	0	0	0.80
100µmol	698	505	7	3	4	1217		4	0	0	3	0	0	
	603	506	5	1	4	1119		2	0	0	2	0	0	
Total	1301	1011	12	4	8	2336	43.30	6	0	0	5	0	0	0.60
200µmol	927	499	3	2	3	1434		7	0	0	3	0	0	
	963	502	3	2	4	1474		5	0	0	1	0	0	
Total	1890	1001	6	4	7	2908	34.40	12	0	0	4	0	0	1.20
300µmol	1012	505	4	3	3	1527		6	0	0	3	0	0	
	1030	503	5	2	3	1543		5	0	0	2	0	0	
Total	2042	1008	9	5	6	3070	32.83	11	0	0	5	0	0	1.10
400µmol	998	537	5	3	4	1547		6	0	0	2	0	0	
	1003	535	3	1	2	1544		4	0	0	2	0	0	
Total	2001	1072	8	4	6	3091	34.50	10	0	0	4	0	0	0.93
500µmol	1212	509	8	3	3	1735		4	0	0	4	0	0	
	1238	507	6	2	2	1755		5	1	0	2	0	0	
Total	2450	1016	14	5	5	3490	29.11	9	1	0	6	0	0	0.98
600µmol	1053	503	5	2	2	1565		5	0	0	3	1	0	
	1078	509	8	2	2	1599		2	0	0	1	0	0	
Total	2131	1012	13	4	4	3164	32.00	7	0	0	4	1	0	0.69
700µmol	1104	504	4	2	4	1618		5	0	0	5	0	0	
	1111	499	4	3	2	1619		3	0	0	2	0	0	
Total	2215	1003	8	5	6	3237	31.00	8	0	0	7	0	0	0.80

GLYCOCHOLIC ACID AT NEUTRAL PH The in vitro Micronucleus Assay														
Dose of Bile Acid	Cell counts						**% BN Cells**	**Mn in Binucleates**			**Mn in Mononucleates**			**%MicN BN**
	MN	**BN**	**3**	**4**	**4+**	**Total**		**1**	**2**	**3**	**1**	**2**	**3**	
800µmol	1400	502	7	5	2	1916		4	0	0	3	0	0	
	1200	505	3	5	3	1716		4	0	0	1	0	0	
Total	2600	1007	10	10	5	3632	27.73	8	0	0	4	0	0	0.79
900µmol	1200	540	3	3	3	1749		4	0	0	2	0	0	
	1205	574	2	5	3	1789		6	0	0	1	0	0	
Total	2405	1114	5	8	6	3538	31.50	10	0	0	3	0	0	0.90
1000µmol	1154	499	4	4	4	1665		5	0	0	1	0	0	
	1213	513	2	5	1	1734		3	0	0	2	0	0	
Total	2367	1012	6	9	5	3399	29.80	8	0	0	3	0	0	0.80
2000µmol	1598	422	5	2	5	2032		4	1	0	3	0	0	
	1618	549	7	2	4	2180		7	0	0	2	1	0	
Total	3216	971	12	4	9	4212	23.05	11	1	0	5	1	0	1.24
3000µmol	1728	438	5	6	3	2180		3	0	0	4	0	0	
	1789	482	4	4	4	2283		5	0	0	1	0	0	
Total	3517	920	9	10	7	4463	20.61	8	0	0	5	0	0	0.85

DEOXYCHOLIC ACID AT NEUTRAL PH The in vitro Micronucleus Assay														
Dose of Bile Acid	Cell counts						**% BN Cells**	**Mn in Binucleates**			**Mn in Mononucleates**			**%MicN BN**
	MN	**BN**	**3**	**4**	**4+**	**Total**		**1**	**2**	**3**	**1**	**2**	**3**	
00µmol	602	527	4	2	4	1139		5	0	0	2	0	0	
	752	536	3	1	4	1296		2	0	0	1	0	0	
Total	1354	1063	7	3	8	2435	43.66	7	0	0	3	0	0	0.65
10µmol	887	512	4	3	3	1409		5	0	0	1	0	0	
	920	514	5	2	2	1443		5	0	0	1	0	0	
Total	1807	1026	9	5	5	2852	36.00	10	0	0	2	0	0	0.97
25µmol	702	503	3	3	2	1213		7	0	0	4	0	0	
	814	504	3	4	2	1327		5	0	0	1	0	0	
Total	1516	1007	6	7	4	2540	39.65	12	0	0	5	0	0	1.19
50µmol	931	501	3	5	6	1446		7	0	0	4	0	0	
	1019	503	5	2	4	1533		11	0	0	3	0	0	
Total	1950	1004	8	7	10	2979	33.70	18	0	0	7	0	0	1.80
100µmol	1127	503	7	3	5	1645		12	0	0	2	0	0	
	1273	508	4	2	2	1789		9	0	0	3	0	0	
Total	2400	1011	11	5	7	3434	29.44	21	0	0	5	0	0	2.10
200µmol	1527	378	6	3	4	1918		6	0	0	2	0	0	
	1600	412	7	3	7	2029		9	0	0	1	0	0	
Total	3127	790	13	6	11	3947	20.0	16	0	0	3	0	0	2.02
300µmol	1500	312	3	4	2	1821		6	0	0	3	0	0	
	1511	348	6	2	1	1868		4	0	0	3	0	0	
Total	3011	660	9	6	3	3690	17.90	10	0	0	6	0	0	1.52
400µmol	1499	172	3	4	3	1681		5	0	0	3	0	0	
	1521	188	2	4	3	1718		3	0	0	1	0	0	
Total	3020	360	5	8	6	3399	11.00	8	0	0	4	0	0	2.2

CHOLIC ACID AT NEUTRAL PH The in vitro Micronucleus Assay														
Dose of Bile Acid	Cell counts						**% BN Cells**	**Mn in Binucleates**			**Mn in Mononucleates**			**%MicN BN**
	MN	**BN**	**3**	**4**	**4+**	**Total**		**1**	**2**	**3**	**1**	**2**	**3**	
00µmol	515	507	5	2	4	1033		4	0	0	3	0	0	
	505	506	2	1	1	1015		4	0	0	2	0	0	
Total	1020	1013	7	3	5	2048	49.50	8	0	0	5	0	0	0.79
10µmol	548	497	6	3	3	1057		4	0	0	2	0	0	
	561	504	5	4	0	1074		2	0	0	2	0	0	
Total	1109	1001	11	7	3	2131	47.00	6	0	0	4	0	0	0.60
25µmol	726	505	3	2	4	1240		4	0	0	2	0	0	
	780	509	2	1	2	1294		6	0	0	1	0	0	
Total	1506	1014	5	3	6	2534	40.00	10	0	0	3	0	0	0.99
50µmol	738	489	3	1	5	1236		2	0	0	1	0	0	
	751	500	3	1	3	1258		5	0	0	1	0	0	
Total	1489	989	6	2	8	2494	39.70	7	0	0	2	0	0	0.71
100µmol	637	505	2	4	2	1150		4	0	0	3	0	0	
	665	507	1	4	2	1179		3	0	0	1	0	0	
Total	1304	1012	3	8	4	2331	43.42	7	0	0	4	0	0	0.69
200µmol	899	502	6	1	5	1413		5	0	0	3	0	0	
	910	496	5	2	1	1414		5	0	0	2	0	0	
Total	1809	998	11	3	6	2827	35.30	10	0	0	5	0	0	1.00
300µmol	998	507	4	4	2	1515		4	0	0	1	0	0	
	1020	504	2	7	1	1534		8	0	0	3	0	0	
Total	2018	1011	6	11	3	3049	33.20	12	0	0	4	0	0	1.20
400µmol	1327	457	6	3	1	1794		3	0	0	3	0	0	
	1373	434	8	1	2	1818		3	0	0	2	0	0	
Total	2700	890	13	4	3	3610	24.65	6	0	0	5	0	0	0.67
500µmol	1217	247	6	2	3	1475		2	0	0	0	0	0	
	1243	260	3	2	4	1512		2	0	0	1	0	0	
Total	2460	507	9	4	7	2987	17.00	4	0	0	1	0	0	0.79

TAURO DEOXYCHOLIC ACID AT NEUTRAL PH The in vitro Micronucleus Assay														
Dose of Bile Acid	Cell counts						**% BN Cells**	**Mn in Binucleates**			**Mn in Mononucleates**			**%MicN BN**
	MN	**BN**	**3**	**4**	**4+**	**Total**		**1**	**2**	**3**	**1**	**2**	**3**	
00µmol	599	504	3	3	5	1114		3	0	0	3	0	0	
	607	508	3	0	3	1121		5	0	0	2	0	0	
Total	1206	1012	6	3	8	2235	45.28	8	0	0	5	0	0	0.79
100µmol	572	499	5	5	3	1084		5	0	0	3	0	0	
	588	501	3	2	2	1096		2	0	0	0	0	0	
Total	1160	1000	8	7	5	2179	45.89	7	0	0	3	0	0	0.70
200µmol	602	517	3	2	4	1128		4	0	0	2	0	0	
	632	508	2	1	3	1146		1	0	0	2	0	0	
Total	1234	1025	5	3	7	2274	45.10	5	0	0	4	0	0	0.49
300µmol	511	497	3	3	3	1017		2	0	0	3	0	0	
	508	503	0	4	2	1017		5	0	0	2	0	0	
Total	1019	1000	3	7	5	2034	49.16	7	0	0	5	0	0	0.70
400µmol	642	496	6	2	2	1148		6	0	0	2	0	0	
	658	505	5	1	2	1155		5	0	0	2	0	0	
Total	1300	1001	11	3	4	2319	43.2	11	0	0	4	0	0	1.10
500µmol	589	486	4	4	3	1086		6	0	0	2	0	0	
	611	508	2	8	1	1130		3	1	0	1	0	0	
Total	1200	994	6	12	4	2216	44.90	9	1	0	3	0	0	1.01
600µmol	886	490	3	2	3	1384		4	1	0	3	0	0	
	914	499	4	0	2	1419		4	0	0	2	0	0	
Total	1800	989	7	2	5	2803	35.28	8	1	0	5	0	0	0.91
700µmol	742	505	4	0	4	1255		3	0	0	3	0	0	
	758	510	4	3	4	1279		8	0	0	3	0	0	
Total	1500	1015	8	3	8	2534	40.10	11	0	0	6	0	0	1.10

TAURODEOXYCHOLIC ACID AT NEUTRAL PH The in vitro Micronucleus Assay														
Dose of Bile Acid	Cell counts						**% BN Cells**	**Mn in Binucleates**			**Mn in Mononucleates**			**%MicN BN**
	MN	**BN**	**3**	**4**	**4+**	**Total**		**1**	**2**	**3**	**1**	**2**	**3**	
800µmol	1001	490	2	4	3	1500		5	0	0	2	0	0	
	1012	507	1	1	3	1524		2	0	0	1	0	0	
Total	2013	997	3	5	6	3024	33.00	7	0	0	3	0	0	0.70
900µmol	1212	500	3	3	5	1723		4	0	0	2	0	0	
	1238	519	2	4	3	1766		3	0	0	2	0	0	
Total	2450	1019	5	7	8	3489	29.21	7	0	0	4	0	0	0.69
1000µmol	1098	497	6	6	4	1611		4	0	0	4	0	0	
	1118	493	4	7	2	1624		4	0	0	2	0	0	
Total	2216	990	10	13	6	3235	30.60	8	0	0	6	0	0	0.81
2000µmol	1497	425	3	3	5	1933		3	0	0	2	0	0	
	1521	435	2	0	2	1960		2	0	0	1	0	0	
Total	3018	860	5	3	7	3893	22.10	5	0	0	3	0	0	0.58
3000µmol														
Total														

GLYCO DEOXYCHOLIC ACID AT NEUTRAL PH The in vitro Micronucleus Assay														
Dose of Bile Acid	Cell counts						**% BN Cells**	**Mn in Binucleates**			**Mn in Mononucleates**			**%MicN BN**
	MN	**BN**	**3**	**4**	**4+**	**Total**		**1**	**2**	**3**	**1**	**2**	**3**	
00µmol	1050	960	10	4	6	2030	47.29	8	0	0	4	0	0	0.83
	1016	954	8	6	5	1989	47.96	5	0	1	3	0	1	0.63
Total	2066	1914	18	10	11	4019	47.625	13	0	1	7	0	1	0.73
100µmol	1401	972	6	3	2	2384	40.77	4	2	0	7	0	0	0.62
	1360	980	5	1	1	2347	41.77	6	1	0	4	0	0	0.71
Total	2761	1952	11	4	3	4731	41.27	10	3	0	11	0	0	0.665
500µmol	1560	1002	5	3	1	2571	38.97	6	1	0	5	0	1	0.70
	1586	1010	4	2	2	2604	38.79	5	2	0	3	2	0	0.69
Total	3146	2012	9	5	3	5175	38.88	11	3	0	8	2	1	0.695
1000µmol	1708	1015	5	1	4	2733	37.14	4	2	0	5	1	0	0.59
	1682	956	6	3	2	2649	36.09	7	1	0	3	2	0	0.84
Total	3390	1971	11	4	6	5382	36.615	11	3	0	8	3	0	0.715
2000µmol	2301	1014	4	5	1	3325	30.50	5	1	0	3	1	0	0.59
	2217	982	3	4	2	3208	30.6	4	3	0	6	1	0	0.71
Total	4518	1996	7	9	3	6533	30.55	9	4	0	9	2	0	0.65

MITOMYCIN C AT NEUTRAL PH The in vitro Micronucleus Assay														
Dose of Bile Acid	Cell counts						**% BN Cells**	**Mn in Binucleates**			**Mn in Mononucleates**			**%MicN BN**
	MN	**BN**	**3**	**4**	**4+**	**Total**		**1**	**2**	**3**	**1**	**2**	**3**	
00µmol	597	547	2	1	3	1150		5	0	0	3	0	0	
	603	555	1	1	3	1163		3	0	0	1	0	0	
Total	1200	1102	3	2	6	2313	47.64	8	0	0	4	0	0	0.73
10mol	493	509	5	3	2	1012		6	0	0	5	0	0	
	505	507	2	2	1	1017		4	1	0	2	0	0	
Total	998	1016	7	5	3	2029	50.10	10	1	0	7	0	0	1.08
30mol	668	518	2	5	5	1198		12	1	0	2	0	0	
	677	505	2	4	1	1189		8	0	1	4	0	0	
Total	1345	1023	4	9	6	2387	42.90	20	1	1	6	0	0	2.15
50µmol	1605	987	8	3	7	2610	37.82	36	3	0	9	0	0	3.95
	1312	1015	4	11	3	2345	43.28	32	1	1	6	0	0	3.35
Total	2917	2002	12	14	10	4955	40.55	68	4	1	15	0	0	3.65
70µmol	599	476	5	1	2	1083		17	1	0	3	0	0	
	608	484	2	1	2	1097		21	1	0	2	0	0	
Total	1207	960	7	2	4	2180	44.91	38	2	0	5	0	0	4.17
100µmol	901	447	3	3	3	1357		14	1	0	4	0	0	
	909	473	1	4	0	1387		19	0	0	3	0	0	
Total	1810	920	4	7	3	2744	33.53	33	1	0	7	0	0	3.70

Appendix C: Raw data from MN analysis at pH 5.5

Table A4.8 MICRONUCLEUS INDUCTION BY TAUROCHOLIC ACID AT pH 5.5- 6														
DOSE µmol	Cell counts						% BN Cells	Micronucleated cells in Binucleates			Micronucleted cells in mononucleates			% MicN BN
	MN	BN	3	4	4	Total		1	2	3	1	2	3	
00	1890	1000	3	4	3	2900	34.48	8	0	0	5	1	0	0.80
00	1970	1000	4	1	2	2977	33.59	10	1	0	5	0	0	1.10
	3860	2000	7	5	5	5877	34.03							0.95
100µmol	2010	1000	2	1	4	3017	33.15	7	0	0	4	0	0	0.70
100µmol	1718	1000	3	2	5	2728	36.66	8	1	0	5	2	0	0.90
	3728	2000	5	3	9	5745	34.81							0.80
500µmol	2307	786	3	1	2	3099	25.36	6	0	0	6	0	0	0.76
500µmol	2145	810	2	3	4	2964	27.33	8	1	1	5	2	1	1.24
	4452	1596	5	4	6	6063	26.32							1.00
1000µmol	2765	550	3	1	3	3322	16.56	7	0	0	4	0	0	1.27
1000µmol	2359	400	1	2	1	2763	14.48	5	0	0	3	0	0	1.25
	5124	950	4	3	4	6085	15.61							1.26
N0RMAL CONTROL	1210	1000	1	3	2	2216	45.13	5	1	0	5	1	1	0.60

Table A4.9 MICRONUCLEUS INDUCTION BY GLYCOCHOLIC ACID AT pH 5.5- 6														
DOSE µmol	Cell counts						% BN Cells	Micronucleated cells in Binucleates			Micronucleted cells in mononucleates			% MicN BN
	MN	BN	3	4	4	Total		1	2	3		2	3	
00	1700	1000	2	1	3	2706	36.95	7	2	1	4	0	0	1.00
00	1689	938	3	1	3	2634	35.61	8	1	0	6	0	0	0.96
							36.29							0.98
100µmol	2109	1000	1	1	3	3114	32.11	6	0	0	4	0	0	0.60
100µmol	1820	960	2	4	2	2788	34.43	7	1	0	3	0	0	0.83

							33.21							0.72
500µmol	2156	690	4	1	2	2853	24.19	5	2	0	5	0	0	1.02
500µmol	2012	742	1	3	3	2761	26.87	3	0	0	4	0	0	0.40
							25.51							0.71
1000µmol	2570	465	2	1	3	3041	15.29	4	1	0	6	0	0	1.08
1000µmol	2359	378	3	2	1	2743	13.78	3	1	0	3	0	0	1.06
							14.58							1.07
N0RMAL CONTROL	1089	1000	1	3	2	2095	47.73	5	1	1	4	0	0	0.70

Table A4.10 MICRONUCLEUS INDUCTION BY DEOXYCHOLIC ACID AT pH 5.5- 6

DOSE µmol	Cell counts						% BN Cells	Micronucleated cells in Binucleates			Micronucleted cells in Mononucleates			% MicN BN
	MN	BN	3	4	4	Total		1	2	3	1	2	3	
00	1806	987	3	1	4	2801	35.24	12	0	0	8	0	0	1.22
00	1589	1000	4	1	1	2595	38.54	7	0	0	5	0	0	0.70
						5396	36.82							0.96
50µmol	1978	602	1	1	3	2585	23.29	7	0	0	5	0	0	1.16
50µmol	2021	510	2	4	2	2539	20.09	10	0	0	8	0	0	1.96
						5124	21.70							1.53
100µmol	2657	370	3	1	2	3033	12.19	10	0	0	4	0	0	2.70
100µmol	2321	415	1	0	3	2740	15.15	11	0	0	5	0	0	2.65
						5773	13.59							2.68
200µmol	2570	210	0	1	3	2784	7.54	7	0	0	3	0	0	3.33
200µmol	2615	380	2	4	1	3002	12.66	10	0	0	7	0	0	1.84
						5786	10.19							2.88

Table A4.11 MICRONUCLEUS INDUCTION BY CHOLIC ACID AT pH 5.5- 6														
BILE ACID DOSE	Cell counts						% BN Cells	Micronucleated cells in Binucleates			Micronucleted cells in Mononucleates			% MicN BN
	MN	BN	3	4	4	Total		1	2	3	1	2	3	
00	1615	938	2	1	3	2559	36.65	5	0	0	6	0	0	0.53
00	1970	1000	1	0	4	2975	33.61	9	0	0	5	0	0	0.90
							35.02							0.72
50µmol	1865	898	1	0	1	2765	32.48	10	0	0	5	0	0	1.11
50µmol	2000	967	1	3	5	2976	32.49	4	0	0	7	0	0	0.41
							32.49							0.76
100µmol	2372	804	3	4	0	3183	25.26	7	0	0	6	0	0	0.87
100µmol	2420	769	1	1	3	3194	24.08	5	0	0	7	0	0	0.65
							24.7							0.76
200µmol	2564	370	1	1	4	2940	12.59	4	1	0	3	0	0	1.35
200µmol	2147	451	2	3	0	2603	17.33	3	1	0	4	0	0	0.89
							14.81							1.09
500µmol	2780	237	2	0	4	3023	7.84	4	1	0	2	0	0	2.11
500µmol	2489	278	1	3	0	2771	10.03	2	0	0	3	0	0	0.72
							8.9							1.36

Table A4.12 MICRONUCLEUS INDUCTION BY TAURODEOXYCHOLIC ACID AT pH 5.5- 6														
DOSE µmol	Cell counts						% BN Cells	Micronucleated cells in Binucleates			Micronucleted cells in Mononucleates			% MicN BN
	MN	BN	3	4	4	Total		1	2	3	1	2	3	
00	1760	900	3	5	4	2672	33.68	7	0	1	5	1	1	0.88
00	2214	1000	6	2	1	3223	31.02	9	1	2	6	0	1	1.2
							32.35							1.04

100µmol	2076	1000	7	1	2	3086	32.40	4	1	0	5	1	0	0.50
100µmol	1993	800	4	3	1	2801	28.56	8	2	0	7	2	0	1.25
							30.48							0.88
500µmol	2405	600	5	4	1	3015	19.90	5	1	0	6	1	0	1.00
500µmol	2317	517	3	2	4	2843	18.18	6	1	0	5	1	0	1.35
							19.04							1.18
1000µmol	2780	400	5	1	2	3188	12.54	6	0	0	5	1	0	1.4
1000µmol	2317	450	4	2	3	3361	13.38	3	1	0	6	1	0	0.89
							12.96							1.15
Control(pH7.4)	1020	989	4	3	3	2019	48.98	6	1	1	6	1	0	0.81

Table A4.13 MICRONUCLEUS INDUCTION BY GLYCODEOXYCHOLIC ACID AT pH 5.5- 6

DOSE µmol	Cell counts						% BN Cells	Micronucleated cells in Binucleates			Micronucleted cells in Mononucleates			% MicN BN
	MN	BN	3	4	4	Total		1	2	3	1	2	3	
00	1892	986	4	3	2	2887	34.15	9	1	0	6	1	0	1.01
00	2300	1000	3	3	1	3307	30.24	8	2	1	5	1	1	1.10
							32.06							1.05
100µmol	2430	993	5	1	3	3432	28.93	8	2	1	5	2	0	1.11
100µmol	2124	756	3	2	2	2887	26.19	5	2	0	7	0	0	0.79
							27.68							0.95
500µmol	2373	450	7	2	4	2836	15.87	5	1	0	3	1	0	1.33
500µmol	2761	375	4	2	4	3146	11.92	4	2	0	6	1	0	1.60
							13.79							1.47
1000µmol	3012	260	5	2	4	3283	7.92	4	0	0	7	1	0	1.54
1000µmol	2736	167	6	2	5	2916	5.73	2	1	0	8	2	0	1.79
							6.89							1.67
Control(pH7.4)	1127	1002	7	5	2	2143	46.76	8	1	0	6	1	0	0.89

Appendix D: Raw data from necrosis and apoptosis at pH 7.4

Table A4.14 NECROSIS / APOPTOSIS: TAUROCHOLIC ACID AT pH 7.4

DOSE	MONONUC	BINUC	3	4	4+	NECR	%NECR	APOPT	%APOP
000µmol	318	248	1	3	0	14	2.39	3	0.51
100µmol	304	220	2	2	1	12	2.21	2	0.37
300µmol	308	234	4	1	1	18	3.16	4	0.70
500µmol	336	207	1	2	2	20	3.5	2	0.35
700µmol	323	189	2	4	0	40	7.18	4	0.71
1mmol	380	103	1	3	0	81	14.14	5	0.87
2mmol	397	98	2	0	1	86	14.65	3	0.51
3mmol	391	102	1	2	1	98	16.36	4	0.67

Table A4.15 NECROSIS / APOPTOSIS: GLYCOCHOLIC ACID AT pH 7.4

DOSE	MONONUC	BINUC	3	4	4+	NECR	%NECR	APOPT	%APOP
000µmol	260	274	1	0	3	17	3.04	4	0.70
100µmol	276	245		1	0	16	2.95	2	0.37
300µmol	255	280	1	1	3	21	3.71	5	0.88
500µmol	287	230	2	2	0	32	5.76	2	0.36
700µmol	320	189	1	4	0	34	6.17	3	0.54
1mmol	369	110	1	2	1	50	9.27	6	1.10
2mmol	339	127	2	0	1	67	12.36	6	1.10
3mmol	381	160	3	0	2	106	16.13	5	0.76

Table A4.16 NECROSIS / APOPTOSIS: DEOXYCHOLIC ACID AT pH 7.4

DOSE	MONONUC	BINUC	3	4	4+	NECR	%NECR	APOPT	%APOP
000µmol	280	230	1	3	0	11	2.10	3	0.57
10µmol	270	241	1	0	1	14	2.64	4	0.75
25µmol	291	219	2	1	0	22	4.05	8	1.47
50µmol	245	243	1	3	2	42	7.7	11	2.01
100µmol	285	189	1	2	0	82	14.13	21	3.62
200µmol	356	202	2	1	3	86	12.90	18	2.70
300µmol	441	110	3	0	1	73	11.38	13	2.028
400µmol	473	68	2	1	1	105	15.98	7	1.06

Table A4.17 NECROSIS / APOPTOSIS: CHOLIC ACID AT pH 7.4

DOSE	MONONUC	BINUC	3	4	4+	NECR	%NECR	APOPT	%APOP
00µmol	253	264	1	0	2	17	3.14	4	0.74
10µmol	249	270	1	3	1	15	2.76	4	0.74
25µmol	260	237	3	0	3	13	2.50	5	0.97
50µmol	281	241	4	0	2	20	3.64	2	0.36
100µmol	273	219	3	3	2	36	6.62	8	1.47
200µmol	302	198	1	0	3	37	6.72	10	1.82
300µmol	365	101	3	1	2	33	6.47	5	0.98
400µmol	352	136	1	3	1	70	12.32	5	0.88
500µmol	389	120	3	1	0	63	10.88	3	0.52

Table A4.18 NECROSIS / APOPTOSIS: GDCA AT pH 7.4

DOSE	MONONUC	BINUC	3	4	4+	NECR	%NECR	APOPT	%APOP
000µmol	268	238	2	1	1	16	3.01	5	0.94
100µmol	250	246	3	2	0	10	1.93	6	1.16
300µmol	289	237	0	2	2	28	4.99	3	0.53
500µmol	296	220	3	2	0	48	8.29	10	1.73
700µmol	280	187	1	3	2	66	12.11	6	1.10
1mmol	320	190	1	2	3	112	17.67	6	0.95

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Table A4.19 NECROSIS / APOPTOSIS: TDCA AT pH 7.4

DOSE	MONONUC	BINUC	3	4	4+	NECR	%NECR	APOPT	%APOP
000	302	284	1	2	1	11	1.82	5	0.83
100µmol	266	238	1	2	1	8	1.54	4	0.77
300µmol	298	251	0	3	1	25	4.30	3	0.52
500µmol	320	206	3	3	0	64	10.7	4	0.67
700µmol	380	220	1	4	1	54	8.10	10	1.50
1mmol	320	189	1	5	2	111	20.82	5	0.94

Table A4.20 NECROSIS / APOPTOSIS: MITOMYCIN

DOSE nmol	MONONUC	BINUC	3	4	4+	NECR	%NECR	APOPT	%APOP
00	253	264	1	0	2	17	3.14	4	0.74

30	249	270	1	3	1	15	2.76	4	0.74
50	260	237	3	0	3	13	2.50	5	0.97
70	281	241	4	0	2	20	3.64	2	0.36
100	273	219	3	3	2	36	6.62	8	1.47

Appendix E: Raw data from necrosis and apoptosis at pH 5.5

Table A4.21 NECROSIS / APOPTOSIS: TAUROCHOLIC ACID AT pH 5.5

DOSE	MONONUC	BINUC	3	4	4+	NECR	%NECR	APOPT	%APOP
000µmol	260	185	2	3	1	48	9.52	5	0.99
100µmol	258	173	1	2	1	86	16.22	9	1.69
500µmol	280	68	1	1	3	142	28.23	8	1.59
1000µmol	236	82	2	2	1	170	33.66	12	2.38

Table A4.22 NECROSIS / APOPTOSIS: GLYCOCHOLIC ACID AT pH 5.5

DOSE	MONONUC	BINUC	3	4	4+	NECR	%NECR	APOPT	%APOP
000µmol	301	140	2	2	1	62	12.04	7	1.36
100µmol	312	87	1	3	1	112	21.52	11	2.09
500µmol	270	74	1	1	2	163	31.41	8	1.54
1000µmol	281	55	1	3	0	158	31.16	9	1.76

Table A4.23 NECROSIS / APOPTOSIS: DEOXYCHOLIC ACID AT pH 5.5

DOSE	MONONUC	BINUC	3	4	4+	NECR	%NECR	APOPT	%APOP
000µmol	238	216	1	4	1	42	8.27	6	1.18
50µmol	245	156	2	3	1	106	20.19	12	2.29
100µmol	230	112	1	0	3	177	32.84	16	2.97
200µmol	264	78	1	1	2	158	30.50	14	2.70

Table A4.24 NECROSIS / APOPTOSIS: CHOLIC ACID AT pH 5.5

DOSE	MONONUC	BINUC	3	4	4+	NECR	%NECR	APOPT	%APOP
00µmol	245	190	3	2	0	59	11.66	7	1.38
50µmol	260	145	1	3	1	96	18.79	5	0.98
100µmol	207	148	3	1	3	144	28.02	8	1.56
200µmol	224	136	2	3	0	129	25.55	11	2.18
500µmol	226	97	3	1	1	187	35.89	6	1.15

Table A4.25 NECROSIS / APOPTOSIS: TDCA AT Ph 5.5

DOSE	MONONUC	BINUC	3	4	4+	NECR	%NECR	APOPT	%APOP
000	321	171	2	3	0	45	8.17	9	1.63
100µmol	308	142	2	1	2	80	14.76	7	1.29
500µmol	214	94	1	3	0	190	37.25	8	1.57
1000µmol	252	96	1	2	1	150	29.53	6	1.18

Table A4.26 NECROSIS / APOPTOSIS: GDCA AT pH 5.5

DOSE	MONONUC	BINUC	3	4	4+	NECR	%NECR	APOPT	%APOP
000	289	169	3	2	2	54	10.31	5	0.95
100µmol	306	158	1	2	1	76	13.74	8	1.45
500µmol	268	84	1	2	0	160	30.36	12	2.28
1000µmol	256	96	2	3	1	178	32.72	8	1.47