CHAPTER II

2.0 REVIEW OF LITERATURE

2.1. CANCER

2.1.1. INTRODUCTION

Cancer is one of the leading causes of adult deaths worldwide. In India, the International Agency for Research on Cancer estimated indirectly that about 6,35,000 people died from cancer in 2008, representing about 8% of all estimated global cancer deaths and about 6% of all deaths in India. The absolute number of cancer deaths in India is projected to increase because of population growth and increasing life expectancy. Rates of cancer deaths are expected to rise, particularly, from increases in the age specific cancer risks of tobacco smoking, which increase the incidence of several types of cancer. India is a culturally diverse country, with huge regional and rural-to-urban variation in lifestyles and in age-specific adult death rates. (Ferlay et al, 2010; Jha P, 2009).

Human beings and other animals have had cancer throughout recorded history. So it’s no surprise that from the dawn of history people have written about cancer. Some of the earliest evidence of cancer is found among fossilized bone tumors, human mummies in ancient Egypt and ancient manuscripts. Growths suggestive of the bone cancer called osteosarcoma have been seen in mummies. Bony skull destruction as seen in cancer of the head and neck has
been found too. Our oldest description of cancer was discovered in Egypt and dates back to about 3000 BC. It is called the Edwin Smith Papyrus and is a copy of part of an ancient Egyptian textbook on trauma surgery. It describes 8 cases of tumors or ulcers of the breast that were treated by cauterization with a tool called the fire drill. The writing says about the disease, “There is no treatment.” (Devita and Rosenberg, 2012)

The origin of the word cancer is credited to the Greek physician Hippocrates (460-370 BC), who is considered the “Father of Medicine.” Hippocrates used the terms *carcinos* and *carcinoma* to describe non ulcer forming and ulcer forming tumors. In Greek, these words refer to a crab, most likely applied to the disease because the finger like spreading projections from a cancer called to mind the shape of a crab. The Roman physician, Celsus (28-50 BC), later translated the Greek term into *cancer*, the Latin word for crab. Galen (130-200 AD), another Roman physician used the word *oncos* (Greek for swelling) to describe tumors. Although the crab analogy of Hippocrates and Celsus is still used to describe malignant tumors, Galen’s term is now used as a part of the name for cancer specialists oncologists (Gallucci BB, 1985).

During the Renaissance, beginning in the 15th century scientists developed greater understanding of the human body. Scientists like Galileo and Newton began to use the scientific method which later was used to study disease. Autopsies done by Harvey (1628). Led to an understanding of the
circulation of blood through the heart and body that had until then been a mystery. In 1761, Giovanni Morgagni of Padua was the first to do something which has become routine today he did autopsies to relate the patient’s illness to pathologic findings after death. This laid the foundation for scientific oncology the study of cancer. The famous Scottish surgeon John Hunter (1728–1793) suggested that some cancers might be cured by surgery and described how the surgeon might decide which cancers to operate on. If the tumor had not invaded nearby tissue and was “moveable” he said “There is no impropriety in removing it.” A century later the development of anesthesia allowed surgery to flourish and classic cancer operations such as the radical mastectomy were developed (Diamandopoulos GT, 1996).

The 19th century saw the birth of scientific oncology with use of the modern microscope in studying diseased tissues. Rudolf Virchow, often called the founder of cellular pathology, provided the scientific basis for the modern pathologic study of cancer. As Morgagni had linked autopsy findings seen with the unaided eye with the clinical course of illness, so Virchow correlated microscopic pathology to illness. This method not only allowed a better understanding of the damage cancer had done, but also aided the development of cancer surgery. Body tissues removed by the surgeon could now be examined and a precise diagnosis could be made. The pathologist could also tell the surgeon whether the operation had completely removed the cancer (Hajdu SI.A, 2011).
In a normal cell, when DNA gets damaged the cell either repairs the damage or the cell dies. In cancer cells, the damaged DNA is not repaired, but the cell doesn’t die like it should. Instead, this cell goes on making new cells that the body does not need. These new cells will all have the same damaged DNA as the first cell does. People can inherit damaged DNA, but most DNA damage is caused by mistakes that happen while the normal cell is reproducing or by something in our environment. Sometimes the cause of the DNA damage is something obvious, like cigarette smoking (American Cancer Society, 2012).

In most cases the cancer cells form a tumor. Some cancers, like leukemia, rarely form tumors. Instead, these cancer cells involve the blood and blood-forming organs and circulate through other tissues where they grow. Cancer cells often travel to other parts of the body, where they begin to grow and form new tumors that replace normal tissue. This process is called metastasis. It happens when the cancer cells get into the bloodstream or lymph vessels of our body. (American Cancer Society, 2012).

2.1.2. INCIDENCE OF CANCER

Cancer is a dreadful disease and remains a cause of continuing public and scientific concern (Behari and Paulaj 2006). The population of India is now over a billion with an estimated 1.5 million cases of cancer diagnosed per year. Incidence and mortality data for all ages. 5-year prevalence for adult population
only Age standard rate (ASR) and proportions per 1,00,000 showed the following table (Table 1 & 2) (Globocon 2008).

Cancer incidence in Indian men is about half to one third of the incidence recorded in the US and Europe. Incidence rates in Indian women are about half the experience of American and European women. It is estimated that on an average about one in 15 men and one in about 12 women in the urban centers could develop cancer in their life time. The different life expectancy in India and USA plays important role in cancer development. There are higher rates of mortality of people of all ages in India. This perhaps leads to a selective pressure on the whole population and only resistant individuals survive. People in USA have bigger body mass index. There is a group of proteins that are involved in gene expression regulation at the level of chromatin remodeling (histone acetylation). Those proteins perhaps provide the connection between metabolism, ageing and cancer development. They are called sirtuins. High body mass index practically equals metabolic syndrome, which is nothing else than ongoing inflammation which may lead to cancer. (Globocon, 2008).
## Table -1 Estimated incidence, mortality and 5-year prevalence: men

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Incidence</th>
<th>Mortality</th>
<th>5-year prevalence</th>
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<tbody>
<tr>
<td></td>
<td>Number</td>
<td>(%)</td>
<td>ASR (W)</td>
</tr>
<tr>
<td>Stomach</td>
<td>21077</td>
<td>4.9</td>
<td>4.7</td>
</tr>
<tr>
<td>Colorectum</td>
<td>20159</td>
<td>4.7</td>
<td>4.3</td>
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<tr>
<td>Liver</td>
<td>14516</td>
<td>3.4</td>
<td>3.2</td>
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<tr>
<td>Gallbladder</td>
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<td>1.4</td>
<td>1.3</td>
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<tr>
<td>Lung</td>
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<td>10.9</td>
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<tr>
<td>Melanoma of skin</td>
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<td>Prostate</td>
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<tr>
<td>Testis</td>
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</tr>
<tr>
<td>Kidney</td>
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<tr>
<td>Bladder</td>
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<tr>
<td>Leukaemia</td>
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Table 2 - Estimated incidence, mortality and 5-year prevalence: women

<table>
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<th>Cancer</th>
<th>Incidence</th>
<th>Mortality</th>
<th>5-year prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>(%)</td>
<td>ASR (W)</td>
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<tr>
<td>Stomach</td>
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<td>Colorectum</td>
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<td>Liver</td>
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<tr>
<td>Lung</td>
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<td>2.5</td>
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<td>Melanoma of skin</td>
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<td>Ovary</td>
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<td>Kidney</td>
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<td>Bladder</td>
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<tr>
<td>Leukaemia</td>
<td>13816</td>
<td>2.7</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Showing cases per 1 million persons calculated on the basis of current consensus: endometrical cancers include Cervix uteri and corpus uteri GLOBOCAN 2008: Cancer Incidence. Mortality and Prevalence Worldwide Version 1.0 IARC Cancer Base No. 5. Lyon.

IARC Press. 12001
2.2. Bladder Cancer

2.2.1. Introduction

Bladder cancer is the 10th most common cancer worldwide, with the highest rates reported in Europe, North America and Australia and accounting for an estimated 2,61,000 new cases diagnosed and 1,15,000 deaths each year, by comparison, relatively low rates are found in the Far Eastern countries. In Europe, bladder cancer is the 5th most commonly diagnosed cancer type and the 9th leading cause of cancer mortality. It affects men more frequently than women. (Larsson et al, 2008).

More than 60,000 new cases of bladder cancer are diagnosed each year in the United States accounting for approximately 13,000 deaths annually. (Cancer Facts and Figures 2007). In recent decades the overall incidence of bladder cancer has appeared to be rising (Ries LA et al, 1975) and this may be due to the latent effects of tobacco abuse and industrial carcinogens, as well as the overall aging of our population.

When initially diagnosed, most bladder cancers are nonmuscle invasive (also referred to as “superficial”) i.e., either noninvasive and confined to the mucosa or invading the lamina propria but not yet invading the detrusor muscle. In 1999, the American Urological Association (AUA) published a report by Smith and associates on the Bladder Cancer Clinical Guidelines Panel Summary
Report on the Management of Nonmuscle Invasive Bladder Cancer (Stages Ta, T1 and Tis) (AUA Guideline) produced by the AUA’s Bladder Cancer Clinical Guideline Panel (Smith et al, 1999).

That expert panel developed a practice guideline for three types of patients:

- The patient who presents with an abnormal growth on the urothelium but not yet diagnosed with bladder cancer;
- The patient with established bladder cancer of any grade, stage Ta or T1, with or without carcinoma in situ (Tis) who had not had prior intravesical therapies and
- The patient with Tis or high-grade T1 cancer who had at least one course of intravesical therapy. The report provided an evidence-based guideline for the patient with nonmuscle invasive bladder cancer and included management standards, guidelines and options based on the strength of evidence and expected amount of variation in patient preferences.

Bladder cancer usually originates in the bladder lining which consists of a mucous layer of surface cells that expand and deflate (transitional epithelial cells), smooth muscle and a fibrous layer. Tumors are categorized as low stage (superficial) or high stage (muscle invasive) (Smith et al, 1999).
The normal urothelium is composed of 3-7 layers of transitional cell epithelium resting on a basement membrane composed of extracellular matrix (collagen, adhesive glycoproteins, glycosaminoglycans). The epithelial cells vary in appearance. The basal cells are actively proliferating cells resting on the basal membrane; the luminal cells, perhaps the most important feature of normal bladder epithelium, are larger umbrellalike cells that are bound together by tight junctions. Beyond the basement membrane is loose connective tissue, the lamina propria, in which occasionally smooth muscle fibres can be identified. The fibres should be distinguished from deeper, more extensive muscle elements defining the true muscularis propria. The muscle wall of the bladder is composed of muscle bundles coursing in multiple directions. As these converge near the bladder neck, 3 layers can be recognized inner and outer longitudinally oriented layers and a middle, circularly oriented layer.

2.2.2. Histopathological type

2.2.2.1 Transitional cell carcinoma

Approximately 90% of all bladder cancers are transitional cell carcinomas. These tumours most commonly appear as papillary, exophytic lesions, less commonly they may be sessile or ulcerated. Whereas the former group are usually superficial in nature, sessile growths are often invasive.
2.2.2.2 Nontransitional cell carcinomas

2.2.2.2.1 Adenocarcinoma

Adenocarcinomas account for less than 2% of all bladder cancer. Primary adenocarcinomas of the bladder may be preceded by cystitis and metaplasia. Histologically adenocarcinomas are mucus secreting and may have glandular, colloid or signet-ring patterns. Whereas primary adenocarcinomas often arise along the floor of the bladder, adenocarcinomas arising from the urachus occur at the dome. Both tumour types are often localized at the time of diagnosis, but muscle invasion is usually present. Five-year survival is usually less than 40%, despite aggressive surgical management (Wright et al. 1988).

2.2.2.2.2 Squamous cell carcinoma

Squamous cell carcinoma account for between 5% and 10% of all bladder cancers and is often associated with a history of chronic infection, vesical calculi, or chronic catheter use. It may also be associated with bilharzial infection owing to Schistosoma haematobium, because squamous cell carcinoma accounts for approximately 60% of all bladder cancers in Egypt, parts of Africa and Middle East, where this infection is prevalent. These tumours are often nodular and invasive at the time of diagnosis. Histologically they appear as poorly differentiated neoplasms composed of polygonal cells with characteristic intercellular bridges (El-Bolkainy et al. 1981).
2.2.2.2.3 Undifferentiated carcinomas

Undifferentiated bladder carcinomas, which are rare (accounting for less than 2%), have no mature epithelial elements. A small cell type has been described that histologically resembles similar lesions of the lung (Mills et al. 1987).

2.2.2.2.4 Mixed carcinoma

Mixed carcinomas constitute 4-6% of all bladder cancers and are composed of transitional, glandular, squamous or undifferentiated patterns. The most common type comprises transitional and squamous cell elements. Most mixed carcinomas are large and infiltrating at the time of diagnosis (Murphy 1989).

2.2.2.2.5 Rare epithelial and nonepithelial cancers

Rare epithelial carcinomas identified in the bladder include villous adenomas, carcinoid tumours, carcinosarcomas and melanomas. Rare nonepithelial cancers of the urinary bladder include pheochromocytomas, lymphomas, choriocarcinomas and various mesenchymal tumours (hemangioma, osteogenic sarcoma and myosarcoma) (Murphy, 1989).

Cancers of the prostate, cervix and rectum may involve the bladder by direct extension. The most common tumours metastatic to the bladder include (in
order of incidence) melanoma, lymphoma, stomach, breast, kidney and lung (Murphy 1989).

2.2.3. EPIDEMIOLOGY AND RISK FACTORS

2.2.3.1. Epidemiology

Bladder cancer is the 9th most common cancer diagnosis worldwide, with more than 3,30,000 new cases each year and more than 1,30,000 deaths per year, with an estimated male:female ratio of 3.8:1.0. At any point in time 2.7 million people have a history of urinary bladder cancer (Ploeg et al, 2009).

At the initial diagnosis of bladder cancer, 70% of cases are diagnosed as non muscle invasive bladder cancer (NMIBC) and approximately 30% as muscle invasive disease. Among patients treated with radical cystectomy because of MIBC, 57% had muscle invasion at presentation, while 43% had been initially diagnosed with NMIBC that progressed despite organ-preserving treatment (Vaidya et al, 2001).

Approximately one third of patients diagnosed with MIBC have undetected metastasis at the time of treatment of the primary tumour (Prout et al, 1979), while 25% of patients subjected to radical cystectomy present with lymph node involvement at the time of surgery.
2.2.3.2 Tobacco smoking

Tobacco smoking is the most well established risk factor for bladder cancer, causing 50-65% of male cases and 20-30% of female cases. A casual relationship has been established between exposure to tobacco and cancer in studies in which chance, bias and confounding can be ruled out with reasonable confidence (Freedman et al, 2011).

The alleged carcinogenic constituents of tobacco smoke include arylamines, particularly the potent carcinogen 4-aminobiphenyl (4-ABP), polycyclic aromatic hydrocarbons (PAHs), N-nitroso compounds, heterocyclic amines, and various epoxides. The incidence of bladder cancer is directly related to the duration of smoking and number of cigarettes smoked per day (Brennan et al, 2000). The risk of bladder cancer is also higher in those who start smoking at a young age or who are exposed to environmental tobacco smoke during childhood (Bjerregaard et al, 2006). A recent meta-analysis looked at 216 observational studies on cigarette smoking and cancer from 1961 to 2003, with reported estimates for current and/or former smokers. The pooled risk estimates for bladder cancer demonstrated a significant association for both current and former smokers. In an analysis of 21 studies, the overall relative risk calculated for current smokers was 2.77 (95% confidence interval, while an analysis of 15 studies showed that the overall relative risk calculated for former smokers was 1.72 (95% CI) (Gandini et al, 2008). An immediate decrease in the risk of bladder
cancer was observed in those who stopped smoking. The reduction was about 40% within 1-4 years of quitting smoking and 60% after 25 years of cessation (Brennan et al, 2000). The promotion of smoking cessation would result in the incidence of bladder cancer decreasing equally in men and women.

2.2.3.3 Occupational exposure to chemicals

Occupational exposure is the second most important risk factor for bladder cancer. Work related cases accounted for 20-25% of all bladder cancer cases in several series. The substances involved in chemical exposure have been benzene derivatives and amines (2-naphthylamine, 4-ABP, 4,4'-methylenedianiline and o-toluidine), and it is likely to occur in occupations in which dyes, rubbers, textiles, paints, leathers and chemicals are used. The risk of bladder cancer due to occupational exposure to carcinogenic aromatic amines is significantly higher after 10 years, or more; the mean latency period usually exceeds 30 years. These chemicals have contributed minimally to the current incidence of bladder cancer in Western countries because of strict regulations. In fact, there has been a trend towards a decrease in bladder cancer due to occupational exposure, as indicated by a pooled analysis of 11 European case control studies on bladder cancer between 1976 and 1996 (Kogevinas et al, 2003).

An example of occupational exposure is that of aromatic amines. These established carcinogens for urothelium can be inactivated by a metabolic
acetylation pathway. The presence of an NAT2 slow-acetylation genotype has been associated with a higher risk of bladder cancer suggesting that patients who are slow acetylators may be more susceptible to bladder cancer than rapid acetylators. Other risk factors include phenacetin, which was included in 1987 among proven human carcinogens by the International Agency for Research on Cancer (IARC). Some studies have suggested that the risk of bladder cancer due to phenacetin is dose dependent; however, the data concerning its metabolite acetaminophen are controversial (Castelao JE et al 2000).

2.2.3.4 Radiation therapy

Increased rates of secondary bladder malignancies have been reported after external beam radiation therapy (EBRT) for gynaecological malignancies, with relative risks of 2 to 4 (Chrouser et al, 2006). A recent population cohort study identified 2,43,082 men treated for prostate cancer between 1988 and 2003 in the Surveillance, Epidemiology and End Results database (SEER) in the USA. The standardised incidence ratios for bladder cancer developing after radical prostatectomy (RP), EBRT, brachytherapy (BT), and EBRT-BT were 0.99, 1.42, 1.10 and 1.39, respectively, compared with the general US population. The increased risk of bladder cancer in patients undergoing EBRT, BT or ERBT-BT should be taken into account during follow up although the likelihood of mortality was described as very low in a recent study. As bladder cancer requires a long
time to develop, patients treated with radiation and a long life expectancy are at highest risk and should be followed up closely (Nieder et al, 2008).

2.2.3.5 Dietary factors

Several dietary factors had been believed to be related to bladder cancer; however, a link remains controversial. Currently, there is limited evidence of a causal relationship between bladder cancer and dietary factors. A meta-analysis of 38 articles reporting data on diet and bladder cancer supported the hypothesis that vegetable and fruit intake reduced the risk of bladder cancer (Steinmaus et al, 2000). For bladder cancer, there seems to be no association between dietary transfatty acid (TFA) intake and an increased risk, as observed for prostate cancer (Hu et al, 2011).

2.2.3.6 Chronic urinary tract infection

Muscle invasive bladder cancer, particularly invasive squamous cell carcinoma, has been linked to the presence of chronic urinary tract infection (UTI) different from schistosomiasis. A direct association between bladder cancer and UTIs has been observed in several case control studies, reporting a two fold increased risk of bladder cancer in patients with recurrent UTIs in some series. However, some of these results may be attributed to recall bias. Furthermore, to date, no clear relationship between any bacterial or viral infection and bladder cancer has been established in prospective studies (Abol-Enein H, 2008).
2.2.3.7 Chemotherapy

The use of cyclophosphamide, an alkylating agent used for treatment of lymphoproliferative diseases and other non-neoplastic diseases, has been correlated with posterior development of muscle invasive bladder cancer (MIBC) with a period of latency of 6-13 years. Acrolein is a metabolite of cyclophosphamide and is responsible for the increase in the incidence of bladder cancer. This effect occurs independently of the association of haemorrhagic cystitis with the same treatment and counteracted with concomitant application of mercaptoethanesulfonate (Monach et al 2010).

2.2.3.8 Gender

In a retrospective study of patients who underwent radical cystectomy, it was demonstrated that women were more likely to be diagnosed with primary muscle-invasive disease than men (85% vs 51%). It has been proposed that women are more likely to be older than men when diagnosed, with a direct effect on their survival. In addition, delayed diagnosis is more likely in women after haematuria is observed, because the differential diagnosis in women includes diseases more prevalent than bladder cancer (Cárdenas Turanzas et al, 2006). Differences in the gender prevalence of bladder cancer may be due to other factors besides tobacco and chemical exposure. In a large prospective cohort study, post menopausal status was associated with an
increase in bladder cancer risk even after adjusting for smoking status. This result suggests that the differences in oestrogen and androgen levels between men and women could be responsible for some of the difference in the gender prevalence of bladder cancer. Recently a study in Egyptian women was conducted and younger age at menopause (< 45y) was a factor associated with increasing risk of bladder cancer, while multiple pregnancies and use of oral contraceptives were associated with decreased odds of having bladder cancer. The magnitude of associations was higher in the urothelial carcinoma group (Stenzl A, 2010).

2.2.4 DIAGNOSIS

2.2.4.1 Primary diagnosis

- **Symptoms**

  Painless haematuria is a common finding. In addition, some patients complain of urgency, dysuria, increased frequency and pelvic pain. Pelvic pain and all the symptoms related to urinary tract obstruction are found in more advanced tumours.

- **Physical examination**

  Physical examination should include rectal and vaginal bimanual palpation. A palpable pelvic mass can be found in patients with locally advanced tumours. In addition, bimanual examination should be carried out before and
after transurethral resection (TUR) to assess whether there is a palpable mass or if the tumour is fixed to the pelvic wall (Wijkström et al 1998).

2.2.4.2 Bladder imaging

A bladder mass identified by diagnostic imaging such as ultrasonography (US), intravenous urography (IVU), computed tomography (CT) or magnetic resonance (MR) imaging should be confirmed with cystoscopy and histology.

2.2.4.3 Urinary cytology and urinary markers

Examination of a voided urine or bladder-washing specimen for exfoliated cancer cells has high sensitivity in high grade tumours. It is therefore useful when a high-grade malignancy or carcinoma in situ (CIS) is suspected. Positive urinary cytology may indicate a urothelial tumour anywhere in the urinary tract from the calix, through the ureters into the bladder and proximal urethra. Cytological interpretation is user dependent (Raitanen et al, 2002).

The evaluation can be hampered by low cellular yield, urinary tract infections, stones or intravesical instillations. In experienced hands, however, specificity exceeds 90%. Cytology should be performed on fresh urine with adequate fixation. Morning urine is not suitable as cytolysis may often be present. No urinary marker is registered specifically for the diagnosis of invasive bladder cancer. However, as most invasive tumours are of high grade the
positive predictive value of markers may be greater in this setting (Van Rhijn et al, 2005).

2.2.2.4 Cystoscopy

The diagnosis of bladder cancer ultimately depends on cystoscopic examination of the bladder and histological evaluation of the resected tissue. In general, cystoscopy is initially performed in the office, using flexible instruments. If a bladder tumour has been visualised unequivocally in earlier imaging studies, such as Computed tomography (CT), Magnetic resonance (MR) imaging or Ultrasonography (US) a diagnostic cystoscopy may be omitted as the patient will undergo TUR for a histological diagnosis. (Stenzl et al, 2010).

2.2.2.5 CT imaging for local staging of invasive bladder cancer

The advantages of CT include shorter acquisition time, wider coverage in a single breath hold, and lower susceptibility to various patient factors. Computed tomography imaging is unable to differentiate between stages Ta to T3a, but it is useful clinically for detecting invasion into the perivesical fat (T3b) and adjacent organs. The accuracy of CT in determining extravesical tumour extension varies from 55% to 92% (Kundrar et al 2003) and increases with more advanced disease (Kim et al 1994).
2.2.5 Molecular Mechanisms of Urothelial Carcinogenesis

Abnormal metabolic pathways and molecular instabilities may likewise play a role in bladder cancer development and progression. These include:

- Altered metabolism/detoxification of carcinogens, and
- Inherent or acquired genetic abnormalities that may promote tumor development (oncogenes), inhibit tumor cell proliferation (tumor suppressor genes), or impair DNA repair (DNA repair enzymes).

Pathways involved in altered chemical metabolism of exogenous carcinogens have included aberrant cytochrome P450 metabolism (associated genetic defects), glutathione-s-transferase abnormalities, and N-acetyltransferase genetic and metabolic derangements. In addition, DNA abnormalities may be inherent or acquired secondarily to carcinogenic exposure. Genetic instability may result in abnormal activity of oncogenes (e.g., ras and myc families) resulting in aberrant protein expression (e.g., GDP/GTP binding proteins), cellular proliferation, and resistance to apoptosis. Abnormalities in tumor suppressor genes associated with bladder cancer have also been well studied and include p53, p21, p16, and Rb (retinoblastoma) tumor suppressor genes that may be mutated or inactivated, and such defects may thereby predispose to cell cycle dysregulation and tumor cell development and progression. Alterations in DNA repair (e.g., ner genes, ber genes, and dsb repair genes) have likewise been associated with polymorphisms that may result...
in bladder urothelial carcinogenesis. Other potential inherent and acquired pathways have also been identified and may also be involved including telomere dysfunction, apoptosis, and cellular inflammation. (Hazara et al 2006).

2.3 Free radicals in Health and Disease

A free radical is defined as any molecular species that contains an unpaired electron in the atomic orbital. Radicals are highly reactive that either donate an electron to or extract an electron from other molecules and therefore behave as oxidants or reductants. As a result of their high reactivity, most radicals have a very short half life (10^-6 seconds or less) in biological systems. The most important free radicals produced in the body are oxygen derivatives, particularly superoxide and the hydroxyl radical. Examples of free radicals and reactive oxygen species include superoxide anion radical, hydroxyl radical, nitric oxide, thyl radical, trichloromethyl radical, hypochlorite radical, hypochlorous acid and also some potentially dangerous non radicals such as hydrogen peroxide, singlet oxygen, hypochlorous acid and ozone. Radical production in the body occurs by both endogenous and environmental factors (Halliwell and Gutteridge, 1999) (Figure 2).
Figure - 1 Major sources of free radicals in the body and the consequences of free radical damage.
Oxidative stress has been implicated in the etiology of a host of degenerative diseases including cardiovascular disease, diabetes, cancer, alzheimer's disease, neurodegenerative disorders and in aging (Scalbert et al., 2005b).

Since free radicals are causally involved in the disease state, it is believed that antioxidants should be effective in preventing or delaying their occurrence. Indeed, investigations at the cellular, tissue and whole animal level as well as epidemiological studies, strongly support the concept that nutritional antioxidant status is inversely related to the occurrence of free radical mediated diseases (Glantzounis et al., 2005).

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2.4 Lipid peroxidation (LPO)

Lipid peroxidation is a free radical mediated process, which has been implicated in a variety of disease states. It involves the formation and propagation of lipid radicals the uptake of oxygen and rearrangement of double
bonds in unsaturated lipids which eventually results in destruction of membrane lipids (Devasagayam, 1993).

Lipid peroxidation can have both direct and indirect consequences. Normally cellular membranes are selectively permeable, hence allow only certain solutes to pass through. This ability is lost due to lipid peroxidation whose products modify the physical characteristics of biological membranes. Incorporation of LOOH, changes the physical structure of membrane by decreasing the fluidity and increasing permeability. When free fatty acids get damaged, membrane confirmation is lost and may lead to gaps in the membrane. It can also cause cross-links between two fatty acids and proteins. This can eventually lead to change in membrane properties and loss of its bound enzymes. Lipid peroxidation can also result in formation of several toxic byproducts that can attack other cellular targets including DNA away from the site of generation. They can also alter cell signaling or act as toxic second messengers that amplify damage. Such byproducts include 4-hydroxy nonenal (HNE), malondialdehyde (MDA) etc induce apoptosis. They form adduct with DNA and induce mutagenecity and carcinogenicity and induction of apoptosis (Devasagayam and Kamat, 2000).

LPO is a chain reaction initiated by the attack on the membrane lipids by free radicals that has sufficient reactivity to abstract a hydrogen atom from the methylene group. This leaves behind an unpaired electron on the carbon atom.
The carbon radical is stabilized by molecular rearrangement to produce conjugated diene which then reacts with an oxygen molecule to form a peroxyl radicals. Peroxyl radical can form cyclic peroxide and cyclic endoperoxide. Fragmentation of these peroxides leads to formation of cytotoxic aldehydes such as MDA and Hydroxy noneal substances (Thiobarbituric acid reactive substances) which themselves are capable of chemically modifying proteins and DNA (Khanjuria, 1997). The process of lipid peroxidation is shown in Figure 2.

Lipid peroxidation alters the fluidity of biological membranes and then causes cell degradation. The end product of lipid peroxidation-thiobarbituric acid reactive substances (TBARS) polymerizes membrane proteins and phospholipids through schiffs base formation and damages the enzymes system and DNA. Biomembranes and subcellular organelles are the major site of lipid peroxidation (Lunee, 1990).

Oxidative stress arising as a result of an imbalance between free radical production and antioxidant defenses associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acids. Lipoprotein particles or membranes characteristically undergo the process of lipid peroxidation giving rise to a variety of products including short chain aldehydes such as malondialdehyde or 4- hydroxynonenal, alkanes and alkenes, conjugated dienes and a variety of hydroxides and hydroperoxides. Oxidative
FIGURE - 2 FORMATION OF CONJUGATED DIENE AND LIPID PEROXIDES
FROM A POLY UNSATURATED FATTY ACID BY LIPID PEROXIDATION

(Gibananda Ray et al., 2002)
damage to proteins and nucleic acids similarly gives rise to a variety of specific
damage products as a result of modifications of amino acids or nucleotides. Such
oxidative damage might also lead to cellular dysfunction and contribute to the
pathophysiology of a wide variety of diseases (Griffiths et al, 2002).

2.5 Antioxidants

An antioxidant is defined as "any substance that, when present in low
concentrations compared to that of an oxidisable substrate, significantly delays or
inhibits the oxidation of that substrate". The physiological role of antioxidants is to
prevent damage to cellular constituents arising as a consequence of chemical
reactions involving free radicals. (Halliwell, 1995)

An ideal antioxidant should have the following attributes

- No harmful physiological effects.
- Effective in low concentration. Fat-soluble.
- Carry through effect.
- Not contribute an objectionable flavor, odor or color to the food.
- No destruction during processing.
- Readily available.
- Economical
2.5.1 Antioxidant Synergy

Combination of antioxidants is more effective than the sum of the individual effects (Liu, 2004). Combined interaction improves effectiveness in several ways, for example: Vitamin E and C Ascorbate can reduce Vit E, so in a lipid oxidation system Vit E and C together will be more effective than adding the effects of each alone. In biological samples synergy is also referred to as co-antioxidants. Antioxidant synergy is the key to the overall antioxidant defense system of living systems (Closa and Folch-Puy, 2004).

2.5.3 Antioxidant enzymes

2.5.3.1 Catalase

Catalase was the first antioxidant enzyme to be characterized, it catalyses the two stage conversion of hydrogen peroxide to water and oxygen,

\[
\text{Catalase-Fe (III) + H}_2\text{O}_2 \rightarrow A
\]

\[
A + \text{H}_2\text{O}_2 \rightarrow \text{Catalase-Fe (III) + 2H}_2\text{O} + \text{O}_2
\]

Catalase consists of four protein subunits, each containing a haem group and a molecule of NADPH (Kirkman et al, 1999). The rate constant for the reactions described above is extremely high (107 M/sec), implying that it is virtually impossible to saturate the enzyme \textit{in vivo}. Catalase is largely located within cells
in peroxisomes, which also contain most of the enzymes capable of generating hydrogen peroxide.

2.5.3.2 Glutathione peroxidases and glutathione reductase

Glutathione peroxidases catalyze the oxidation of glutathione at the expense of a hydroperoxide, which might be hydrogen peroxide or another species such as a lipid hydroperoxide (Takahashi et al., 1987).

\[
\text{ROOH + 2GSH} \rightarrow \text{GSSG + 2H}_2\text{O + ROH}
\]

Other peroxides, including lipid hydroperoxides, also act as substrates for these enzymes, which might therefore play a role in repairing damage resulting from lipid peroxidation. Glutathione peroxidases require selenium at the active site (Nakane et al., 1998). The predominant subcellular distribution is in the cytosol and mitochondria suggesting that glutathione peroxidase is the main scavenger of hydrogen peroxide in subcellular compartments. The activity of the enzyme is dependent on the constant availability of reduced glutathione (Masella et al., 2005). The ratio of reduced to oxidized glutathione is usually kept very high as a result of the activity of the enzyme glutathione reductase:

\[
\text{GSSG + NADPH + H}^+ \rightarrow 2\text{GSH + NADP}^+
\]

The NADPH required by this enzyme to replenish the supply of reduced glutathione is provided by the pentose phosphate pathway. Glutathione
reductase is a flavine nucleotide dependent enzyme and has a similar tissue distribution to glutathione peroxidase (Rana et al, 2002).

### 2.5.3.3 Superoxide dismutase

The enzyme superoxide dismutases catalyse the dismutation of superoxide to hydrogen peroxide

\[
O_2^- + O_2^- + 2H^+ \rightarrow \text{H}_2\text{O}_2 + O_2
\]

The hydrogen peroxide must then be removed by catalase or glutathione peroxidase, as described above. There are three forms of superoxide dismutase in mammalian tissues, each with a specific subcellular location and different tissue distribution.

i) Copper zinc superoxide dismutase (CuZ\textsubscript{n}SOD): CuZ\textsubscript{n}SOD is found in the cytoplasm and organelles of virtually all mammalian cells (Liou et al, 1993). It has a molecular mass of approximately 32,000 kDa and has two protein subunits, each containing a catalytically active copper and zinc atom.

ii) Manganese superoxide dismutase (Mn\textsubscript{SOD}): Mn\textsubscript{SOD} is found in the mitochondria of almost all cells and has a molecular mass of 40,000 kDa (Abe and Okazaki, 1987). It consists of four protein subunits, each probably containing a single manganese atom. The amino acid sequence of Mn\textsubscript{SOD} is entirely dissimilar to that of CuZ\textsubscript{n}SOD and it is not inhibited
by cyanide, allowing Mn-SOD activity to be distinguished from that of CuZn-SOD in mixtures of the two enzymes.

iii) Extracellular superoxide dismutase (EC-SOD): EC-SOD was described by Marklund 1982 and is a secretory copper and zinc containing SOD distinct from the CuZnSOD described above. EC-SOD is synthesised by only a few cell types, including fibroblasts and endothelial cells.

2.5.3.4 Non-enzymatic antioxidants

Apart from enzymic antioxidants, a spectrum of nonenzymic antioxidants namely vitamin A, E,C and GSH are important in cellular system in curtailing reactive oxygen species (ROS).

2.5.3.4.1 Vitamin C (Ascorbic acid)

Vitamin C is reported to be associated with good scavenging activities in vivo because they are present both intracellularly as well as in the extracellular fluids. Ascorbic acid is a potential scavenger of O$_2^-$ and peroxyl radicals. Vitamin C is a unique antioxidant and it has a specific role in preventing ROS mediated oxidative damage of mammalian tissues (Indu et al., 1995).

Vitamin C interacts directly with radicals like O$_2^-$ and OH- in plasma, thus preventing damage to red cell membrane (Boyer, 1994). It probably assists tocopherol in inhibition of LPO by recycling the tocopherol radical. It is a good
scavenger of many free radicals like $\text{O}_2^-$, $\text{OH}^-$ and various lipid hydroperoxides (Berger et al., 1997).

Vitamin C is an water soluble antioxidant and can also act as a co-antioxidant by regenerating alpha tocopherol (vit E) from the $\alpha$-tocopheroxyl radical produced via scavenging of lipid soluble radicals (Packer, 1997).

### 2.5.3.4.2 Vitamin E

Vitamin E comprises a group of eight naturally related tocopherols most important being $\alpha$ tocopherol. This is one of the most important lipid soluble antioxidant vitamins. It scavenges peroxyl radical intermediates in lipid peroxidation and is responsible for protecting polyunsaturated fatty acid present in cell membrane and low density lipid proteins against lipid peroxidation (Maseio et al., 1991).

Vitamin E is the most effective chain breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation (Sies, 1992). It acts in conjugation with ascorbate and reduced glutathione (GSH). Once the tocopheroxyl radical is formed, it migrates to the membrane surface and is reconverted to $\alpha$-tocopherol by reaction with either ascorbate or GSH. The resulting ascorbate radical can regenerate ascorbate by reaction with GSH (Bandyopadhyaya et al., 1999).
Free radicals generated within the body can react with polyunsaturated fatty acids to cause a chain reaction, which can destroy many lipid molecules in a short period of time. For a molecule to be a good chain breaking antioxidant, it must be able to produce a stable radical by donating a hydrogen to the damaging unstable radical. α-tocopherol, the best chain breaking antioxidant can donate a phenolic hydrogen to a lipid radical (Liebier, 1993).

2.6 CHITOSAN

2.6.1 INTRODUCTION

Polysaccharides are the most abundant of the four major classes of biomolecules, which also include proteins, lipids and nucleic acids. They are often classified on the basis of the sequences and linkages between their main monosaccharide components, as well as the anomeric configuration of linkages, the ring size (furanose or pyranose), the absolute configuration (D or L) and any other substituents present. Certain structural characteristics such as chain conformation and intermolecular associations influence the physicochemical properties of polysaccharides. For example, polysaccharides containing large numbers of hydroxyl groups are often thought of as being hydrophilic. Polysaccharides fill numerous roles in living organisms, such as the storage and transport of energy (e.g. starch and glycogen) and structural components (e.g. cellulose and chitin).
2.6.2 CHITIN

Chitin is widely distributed in nature, mainly as the structural component of the exoskeletons of crustaceans (crab, shrimp, lobster, krill, squid, crawfish and prawn) and insect cuticles, in marine diatoms and algae, as well as in some fungal cell walls. Structurally, it is an insoluble linear mucopolysaccharide consisting of \( N\)-acetyl-\( D\)-glucosamine (GlcNAc) repeat units, linked by \( \beta\)-(1→4) glycosidic bonds. Technically, the structure of chitin is highly related to that of cellulose and may be regarded as cellulose where the hydroxyl [-OH] at the C-2 position is replaced by an acetamido [-N\( \text{HCOCH}_3\)] group (Suzuki S, 2000).

Resources of chitin for industrial processing are crustacean shells and fungal mycelia; however, its commercial production is usually associated with seafood industries, such as shrimp canning. The processing of crustacean shells mainly involves the removal of proteins (deproteinization in a hot basic solution, usually sodium or potassium hydroxide) and calcium carbonate (demineralization, with diluted acid), both present in crustacean shells in high concentrations, encasing the chitin microfibrils (Kumar M, 2000). Chitin has aroused great interest not only as an underutilized resource, but also as a new functional material of high potential in various fields. Several derivatives have been prepared from chitin, but none was as commonly studied on both the academic and industrial level, as chitosan.
2.6.3 NATURE AND SOURCES

Chitosan, discovered by Rouget in 1859, is a technologically important polysaccharide biopolymer. Chemically, it is a high molecular weight linear polycationic heteropolysaccharide consisting of two monosaccharides, \(N\)-acetyl-Dglucosamine and D-glucosamine, linked together by \(\beta-(1\rightarrow4)\) glycosidic bonds, production of chitosan (Figure 3 and 4). The relative amount of the two monosaccharides in chitosan may vary, giving samples of different degrees of deacetylation (75-95%), molecular weights (50-2,000 kDa), viscosities, pKa values, etc. Therefore, the term chitosan does not refer to a uniquely defined compound, it merely refers to a family of copolymers with various fractions of acetylated units (Chawla M, 2001).

Chitosan is primarily produced from chitin by exhaustive alkaline deacetylation, this involves boiling chitin in concentrated alkali for several hours (40–45% sodium hydroxide, 120°C, 1–3 h). Since this \(N\)-deacetylation is almost never complete, chitosan is considered as a partially \(N\)-deacetylated derivative of chitin. Consequently, a sharp distinction between chitin and chitosan on the basis of the degree of \(N\)-deacetylation cannot be drawn. Enzymatic procedures for chitin deacetylation by chitin modifying enzymes were also investigated in the literature. (Rabea et al 2003).
Figure - 3

Preparation of Chitin & Chitosan

Shellfish wastes from food processing (shrimp, squid, crab)

1. Decalcification in dil. aqueous HCl solution (3% to 5% HCl w/v HCl at room temperature)

2. Proteolysis in dil. aqueous NaOH solution (3% to 5% w/v NaOH, 80°C to 900°C for a few hrs. or room temperature overnight)

3. Decolorization in 0.5% KMnO4 aqueous and oxalic acid aqueous or sunshine

4. Deacetylation in hot concentration NaOH solution (40% to 50% w/v NaOH, at 90°C to 120°C for 4 to 5 hrs)

The crude chitosan is dissolved in aqueous 2% w/v acetic acid. Then the insoluble material is removed giving a clear supernatant solution, which is neutralized with NaOH solution resulting in a purified sample of chitosan as a white precipitate. Further purification may be necessary to prepare medical and pharmaceutical-grade chitosan."
Chitosan is also found in nature, such as in cell walls of fungi of the class *Zygomycetes* (Pochanavanich & P and W. Sunthornsuk, 2002), in the green algae *Chlorella* sp., yeast and protozoa as well as in insect cuticles (Singla and Chawla, 2001). Recent advances in fermentation technology suggest that the cultivation of fungi (*Aspergillus niger*) can provide an alternative source of chitosan (Rabea et al, 2003). However, chitosan from both sources differs slightly: whereas the acetyl groups in chitosan produced from crustacean chitin are uniformly distributed along the polymer chain, a chitosan of similar degree of deacetylation isolated from fungal cell walls would possess acetyl residues that are grouped into clusters. In contrast to most of the naturally-occurring polysaccharides, e.g. cellulose, dextran, pectin, alginic acid, agar, agarose and carragenans which are neutral or acidic in nature, chitosan is an example of a highly basic polysaccharide. Its nitrogen content varies from 5 to 8% depending on the extent of deacetylation it is mostly in the form of primary aliphatic amino groups. (Kumar M, 2000).

### 2.6.4 PHYSICOCHEMICAL ASPECTS

As mentioned above, the term “chitosan” describes a heterogenous group of polymers. Chitosan is commercially available from a number of suppliers in various grades of purity, molecular weights and molecular weight distributions, chain lengths, degrees of deacetylation, charge densities and charge distributions, salt forms, viscosities and water retention values. These properties greatly affect its physicochemical characteristics which in turn govern almost all of its applications.
Figure- 4 Chemical structure of chitosan, and its production from chitin.

Chitosan is a (1→4) linked 2-amino 2-deoxy β-D-glucan, prepared from chitin through alkaline hydrolysis of the N-acetyl group.
2.6.5 MOLECULAR WEIGHT (MW)

Although the underlying chemical and physical effects of some of the applications of chitosan and its derivatives are still not known in detail, considerable evidence has been gathered indicating that most of their physiological activities and functional properties depend on their molecular weight (Rabea, et al. 2003).

The molecular weight distribution of a raw chitosan preparation is influenced by variable conditions employed in the deacetylation process, such as time, temperature, concentration and nature of starting material as well as atmospheric conditions (Wu. A.C.M, 1976). Weight-average molecular weights of several hundreds to over one million Dalton are common, with a mean molecular mass of up to 1 MDa, corresponding to a chain length of approximately 5,000 U (Rhoades and Roller 2000). Because of the influence of polymer composition and molecular weight range on the various physicochemical properties of chitosan, it is very important to adequately characterize each batch of polymer produced. The molecular weight of chitosan can be determined by several methods, such as light scattering spectrophotometry, gel permeation chromatography and viscometry (Kumar. M, 2000).

2.6.6 DEGREE OF DEACETYLATION (DD)

An important parameter to examine closely is the degree of deacetylation of chitosan, i.e. the ratio of N-acetyl-D-glucosamine to D-glucosamine structural
units. In chitin, the acetylated units prevail whereas the degree of deacetylation of chitosan is influenced by the preparation procedure for example increasing proportionally with increasing treatment time (Cho et al, 2000). It has an impact on the extent of moisture absorption, charge distribution, intrinsic viscosity and chitosan solubility in aqueous solutions. A number of analytical tools have been used to define the degree of deacetylation, such as FTIR spectroscopy, UV spectrophotometry, 1H-NMR and 13C solid-state NMR spectroscopy, various titration methods, equilibrium dye adsorption, elemental analysis, acid degradation followed by HPLC and thermal analysis (Kumar, M, 2000).

2.6.7 SOLUBILITY

The main difference between chitin and chitosan lies in their solubility. Deacetylation transforms the insoluble chitin into the acid soluble chitosan. Chitosan is therefore said to be chitin that has been N-deacetylated to such an extent that it becomes soluble in dilute aqueous acids (e.g. 0.1 M acetic acid). Pure native chitosan (pKa ≈ 6.3) is insoluble in water in alkaline medium and even in organic solvents. However, watersoluble salts of chitosan may be formed by neutralization with organic acids (e.g. 1-10% aqueous acetic, formic, succinic, lactic, glutamic and malic acids) or inorganic acids such as hydrochloric acid (Singla and Chawla 2001). The pH-dependent solubility of chitosan is attributed to its amino groups (-NH2), which become protonated upon dissolution at pH 6 or below to form cationic amine groups (-NH3+), increasing intermolecular electric
repulsion and resulting in a polycationic soluble polysaccharide with a large number of charged groups on a weight basis. On the other hand, chitosan tends to lose its charge at higher pH and may therefore precipitate from solution due to deprotonation of the amine groups (Singla and Chawla, 2001).

2.6.8 VISCOSITY AND SOLUTION PROPERTIES

One of the most characteristic properties of many polymers, including chitosan, is their ability to form viscous solutions they could therefore function as thickeners, stabilizers, or suspending agents. Chitosan solutions show pseudo plastic and viscoelastic properties their viscosity is affected by chitosan’s degree of deacetylation, molecular weight and concentration, concentration and types of solvents, the prevailing solution pH and ionic strength, as well as temperature. The viscosity range of commercial chitosans (1% acetic acid at 25°C) is from 10 to 1000 mPa·s (Kumar M, 2000).

2.6.9 CHEMICAL REACTIVITY AND DERIVATIZATION

Chitosan possesses three types of reactive functional groups an amino group at the C-2 position of each deacetylated unit as well as primary and secondary hydroxyl-groups at the C-6 and C-3 positions, respectively, of each repeat unit. These reactive groups are readily subjected to chemical derivatization under mild conditions to allow for the manipulation of mechanical
and physicochemical properties, for example improving chitosan’s solubility at neutral pH ranges (Singla and Chawla 2001).

Furthermore, the presence of free amino groups in chitosan permits its conjugation with some drugs, as well as complexing agents (such as ethylene diamine tetra acetic acid, EDTA) (Bernkopschnurch et al, 1999).

2.6.10 PROCESSABILITY

The superior solubility makes chitosan more easily manageable than chitin. It could be easily processed into a variety of useful forms such as gels, membranes, sponges, films, fibers and beads by controlling factors such as acid solvent, degree of deacetylation and molecular weight to address a variety of applications.

2.6.11 MISCELLANEOUS PROPERTIES

At pH < 6.5, chitosan is a promising cationic mucoadhesive polysaccharide. Several factors affect the mucoadhesive properties of chitosan, including its concentration, molecular weight, degree of deacetylation and cross linking, in addition to contact time, environmental pH and ionic strength.

Chitosan and its derivatives have strong film and gel forming properties, with good oxygen/moisture transmission coefficients and substantivity, they are also endowed with permeation and absorption enhancing effects and are able to enhance the dissolution and bioavailability of poorly absorbable drugs thus
lending themselves to a variety of applications. Moreover, they are capable of strongly binding transition metals such as copper, zinc, iron \textit{in vitro} through a chelation process, probably due to their high percentage of nitrogen (6.89\%) (Rabea et al, 2003).

2.6.12 BIOLOGICAL PROPERTIES

Much of the commercial interest in chitosan and its derivatives during the last two decades arises from the fact that they combine several favorable biological characteristics, including biodegradability, biocompatibility and non toxicity, properties which render natural polymers superior over present day synthetic polymers, making them valuable materials for pharmaceutical, biomedical as well as industrial applications.

2.6.13 BIODEGRADATION

Whereas chitosan solutions are highly stable over a long period there is sometimes a need for degrading chitosan to a level suitable for a particular application or as a means of conferring solubility to chitosan at neutral pH. Several methods for producing chitosan oligomers (chitosanolysis) have been described in literature including radiation, chemical (acid hydrolysis or oxidative reductive degradation) and enzymatic methods of which enzymatic degradation preferred since reaction and thus product formation could be controlled by means of pH, temperature and reaction time (Rhoades and Roller 2000).
2.6.14 BIOCOMPATIBILITY

One of the most important biological properties of any implantable biomaterial is biocompatibility i.e. it should not be affected by the host and at the same time should not elicit any undesirable local or systemic effects. Chitosan is well tolerated by living tissues, including the skin, ocular membranes, as well as the nasal epithelium and has thus been proven valuable for a wide range of biomedical applications (Khan et al, 2000).

2.6.15 SAFETY

The low toxicity profile of chitosan compared with other natural polysaccharides is another of its many attractive features. It has been reported that the purity of chitosan influences its toxicological profile, yet its safety in terms of inertness and low or no toxicity has been demonstrated by in vivo toxicity studies (Dodne et al, 1998).

2.6.16 APPLICATIONS

Although extensive resources were involved in both research and development of processes and applications for chitosan, only the last two decades have witnessed serious developments of a variety of technologies based on the commercial utilization of chitosan and its derivatives. Chitosan, its oligomers and a number of its derivatives emerged as new biomaterials and are currently in use or under consideration in a number of applications.
(pharmaceutical, cosmetic, medical, food, textile, agricultural, etc.), summarized in Table 7. Due to the wide scope of applications, only a number of them will be further discussed in this section.

Introduced to the market in the 1990's, chitosan has been the subject of much research regarding its potential as a useful and promising pharmaceutical excipient in various pharmaceutical formulations (Singla and Chawla 2001). Next to the more traditional formulations, chitosan has found use in novel applications such as vaccine delivery, peptide and gene delivery, in addition to its use in tissue engineering. So far, the nasal chitosan vaccine delivery system against influenza has been tested for vaccination in human subjects and has been proven to be both effective and protective (Illum et al, 2001). Chitosan's utility as a pharmaceutical ingredient gained more interest when a scientific understanding of at least some of the pharmacological activities of this versatile carbohydrate began to evolve (Brode et al 1991).

**TABLE 3 - Applications of chitosan**

<table>
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<tr>
<th>Applications</th>
<th>Benefits/ advantage</th>
<th>References</th>
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<td>PHARMACEUTICALS AND COSMETICS</td>
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<tr>
<td>Conventional formulations</td>
<td>binder; disintegrant, coating, lubricant, diluent</td>
<td>(Kofuji et al, 2005)</td>
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<tr>
<td>➢ Tablet manufacture</td>
<td>sustained drug release; enhanced absorption</td>
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<tr>
<td>➢ Gels</td>
<td>controlled drug release</td>
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<td>Membranes</td>
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<td>Ophthalmic formulations</td>
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<td>Transdermal delivery systems</td>
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<td>Colon-specific drug delivery</td>
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<td>Targeted cancer therapy</td>
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<td>Vaccine delivery</td>
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<td>Oral vaccination</td>
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<td>Peptide drug delivery</td>
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<td>Gene and nucleic acid delivery</td>
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<td>Deodorant formulations</td>
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<td>Nonirritating, enhancing fragranc adhesion, deodorizing</td>
<td>(Hohle and Griesbach, 1999)</td>
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<tr>
<td>Hair and skin care products</td>
<td>Preservative, emulgator, thickener, moisturizer, soothing seffect on skin</td>
<td>(Moore. A, 2002)</td>
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### MEDICAL AND BIOMEDICAL

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<tr>
<th>Antacid and anti-ulcerogenic</th>
<th>demulcent and protective effect on stomach mucosa</th>
<th>(Ito et al, 2000)</th>
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<tr>
<td>Antidiabetic (hypoglycemic)</td>
<td>lowering of blood glucose level, increasing glucose tolerance and insulin secretion</td>
<td>(Lee et al, 2003)</td>
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<td>Antihypertensive</td>
<td>-</td>
<td>(Lee et al, 2003)</td>
</tr>
<tr>
<td>Antioxidant</td>
<td>scavenging of radicals and chelation of divalent metals</td>
<td>(Chen et al, 2003)</td>
</tr>
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<td>Antitumor</td>
<td>induction of apoptosis in tumor cells</td>
<td>(Qin et al, 2002)</td>
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<td>Anticoagulant</td>
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<td>(Park et al, 2004)</td>
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<tr>
<td>Hemostatic</td>
<td>biological adhesive for soft tissues</td>
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<td>Spermicidal</td>
<td>strong binding to mammalian cells</td>
<td>(Shigemasa and Minami, 1995)</td>
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<td>Hypcholesterolaemic, nutritional aid for weight loss</td>
<td>prevention of fat absorption, reduction of blood lipid levels</td>
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<td>Wound dressings, products for wound treatment</td>
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<td>Contact and bandage lenses</td>
<td>optical clarity; wound-healing, mechanical stability, sufficient</td>
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<td>optical correction, immunological compatibility, gas permeability, wettability, antimicrobial</td>
<td>(Decker et al, 2005)</td>
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<td><strong>Immunopotentiator</strong></td>
<td>stimulation of immune system, augmenting immunogenicity of coadministered antigens, promoting resistance to systemic infections</td>
<td>(Okawa et al, 2003)</td>
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<td><strong>Surgical sutures and implants</strong></td>
<td>Biodegradable</td>
<td>(Singla and Chawal, 2001)</td>
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<td><strong>Hemodialysis membranes</strong></td>
<td>-</td>
<td>(Shigemasa and Minami, 1995)</td>
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<td><strong>Coating for prosthetics and biomedical devices</strong></td>
<td>Thromboresistance, compatibility with blood, anti-biofilm properties</td>
<td>(Carlson et al, 2008)</td>
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</table>

**TISSUE ENGINEERING**

<p>| <strong>Scaffold for tissue engineering applications</strong> | promoting tissue growth and differentiation | (Dang and Leong, 2006) |
| <strong>Artificial skin grafts (substratum for skin replacement)</strong> | nonantigenic; performs as a biodegradable template for synthesis of neodermal tissue | (Kumar. M, 2000) |</p>
<table>
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<td>Soil and plant revitalizer</td>
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<td>(Mulawarman et al, 2001)</td>
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<tbody>
<tr>
<td>Food processing</td>
<td>enhances safety, quality and shelflife of food, clarification of liquids, preservative, thickener</td>
<td>(Devlieghere et al, 2004)</td>
<td></td>
</tr>
<tr>
<td>Coatings for vegetables, fruits and fish</td>
<td>improving shelf life, preventing moisture loss, delaying fungal growth</td>
<td>(Jeon et al, 2002)</td>
<td></td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th><strong>TEXTILE INDUSTRIES</strong></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Edible antimicrobial films for food packaging</td>
<td>-</td>
<td>(Durango et al, 2006)</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>WASTE WATER TREATMENT</strong></th>
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</thead>
<tbody>
<tr>
<td>Food and beverage processing plants</td>
<td>coagulation and flocculating agent resulting dried sludge used in animal feeds</td>
<td>(Gamage and Shahidi et al, 2007)</td>
<td></td>
</tr>
<tr>
<td>Industrial waste</td>
<td>removal of heavy metal ions through adsorption and chelation</td>
<td>(Paulino et al, 2008)</td>
<td></td>
</tr>
<tr>
<td>Textile effluents</td>
<td>sorption of dyes, due to high affinity to many classes of dyes</td>
<td>(Sye et al, 2008)</td>
<td></td>
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</table>
In spite of the promising use of chitosan in the pharmaceutical industry, however most of the chitosan researches are directed toward medical applications. Unfortunately a survey of the available literature revealed that there are only relatively few specific and objective research studies to support claims, ascribing a range of rather impressive pharmacological properties to this biopolymer. Most of these studies are very difficult to take seriously with little scientific evidence to back them up. For example, chitosan is often being heralded and sold as a “revolutionary” weight loss supplement a “fat magnet” although this presumptive property often discredited in recent studies (Gades et al, 2005).

Some studies showed that chitosan as an immune adjuvant could effectively promote local immune response and enhance antigen presentation (Borges et al, 2005; Xie et al 2007). Porporatto et al (1997), propose the following mechanisms for the modulation of mucosal immune response

<table>
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<th>MISCELLANEOUS</th>
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<tr>
<td>Photographic paper</td>
<td>resistance to abrasion; favorable optical characteristics; film-forming ability</td>
</tr>
<tr>
<td>Paper finishing</td>
<td>imparts wet strength to paper</td>
</tr>
</tbody>
</table>
as a dietary fiber chitosan might have an impact on the intestinal flora and mucosal microenvironment thus influencing local immune function

as a delivery agent it might decrease the clearance rate and stimulate the uptake of antigens

as an adjuvant it might provide “danger signals” being a component of fungal cell walls, possibly through the activation of components of the innate immune system such as macrophages. They therefore conclude that chitosan could be used to modulate the immune response to orally administered antigens.

Probably one of the most prominent commercial applications of chitosan is its use as a hemostatic. Several chitosan based wound dressings are available on the market for clinical use including HemCon® Bandage and ChitoFlex wound dressings (HemCon Medical Technologies Inc., West Yorkshire, UK), as well as CELOX™ (Medtrade Products Ltd., Crewe, England), both claimed to be FDA approved.

2.6.17 ECONOMIC ASPECTS AND REGULATORY STATUS

Since a large amount of the crustacean exoskeleton is readily available as a byproduct of the seafood processing industry, the raw material for chitosan production is relatively inexpensive and thereby the production of chitosan on a large scale from this renewable bio resource is economically feasible. Chitosan is commercially produced in different parts of the world (Japan, North America,
Poland, Italy, Russia, Norway and India) on a large scale. It has been estimated that up to 10^9–10^10 tons of chitosan are annually produced in nature. Another important aspect to be considered is that utilizing the shellfish waste for chitin production provides a solution for the waste disposal problem, and provides an alternative for the use of this oceanic resource. (Rabea et al, 2003)

Generally Recognized As Safe (GRAS) is a designation of the FDA (Food and Drug Administration) in the United States of America, that a chemical or substance added to foods and beverages is considered safe by experts. Chitosan has not been officially proclaimed GRAS by the FDA but one Norwegian company (Primex Ingredients ASA), which manufactures shrimp-derived chitosan, has announced in 2001 that its purified chitosan product (ChitoClear®) has achieved a GRAS self-affirmed status in the U.S. market. On the other hand, the FDA has approved chitosan for medical uses such as bandages and drug encapsulation. Chitosan is also widely used in foods in Italy, Finland, Korea and Japan.

2.7 FILM-FORMING ABILITY

Chitosan with higher molecular weight have been reported to have good film-forming properties as a result of intra and intermolecular hydrogen bonding (Muzzarelli RAA 1977). A patent was granted to G.W. Rigby in 1936 for the earliest attempt to form films from chitosan. These films were described as flexible, tough, transparent and colorless with a tensile strength of about 9,000
psi and prepared by a solvent casting method. Chitosan films prepared by similar methods were reported later by Caner 1998 and others and Wiles, 2000 and others.

These films were described to have good gas barrier and mechanical properties. The chitosan film characteristics, however, varied from one report to another. Differences in the sources of chitin used to produce chitosan, chitosan properties, solvents used, methods of film preparation and types and amounts of plasticizers used affect the quality of the chitosan films (Lim and Wan 1995; Remuñán-López and Bodmeier 1996; Begin and Calsteren 1999; Nunthanid 2001). The film forming ability of chitosan extracted from crawfish has been reported by Nadarajah and Prinyawiwakul, (2002).

2.8 Antimicrobial Activity

The antimicrobial activity of chitin, chitosan and their derivatives against different groups of microorganisms, such as bacteria, yeast, and fungi, has received considerable attention in recent years. Two main mechanisms have been suggested as the cause of the inhibition of microbial cells by chitosan. The interaction with anionic groups on the cell surface, due to its polycationic nature, causes the formation of an impermeable layer around the cell, which prevents the transport of essential solutes. It has been demonstrated by electron microscopy that the site of action is the outer membrane of gram negative bacteria. The permeabilizing effect has been observed at slightly acidic
conditions in which chitosan is protonated, but this permeabilizing effect of chitosan is reversible (Helander et al, 2001).

The second mechanism involves the inhibition of the RNA and protein synthesis by permeation into the cell nucleus. Liu et al (2001) have observed labelled chitosan oligomers with Mw from 8 to 5 kDa inside the *E. coli* cell and they showed good antibacterial activities.

### 2.9 Wound Healing

Slow healing and non-healing wounds, such as ulcers, as well as wounds caused by major or minor injuries, surgery, or burns, represents the most widespread treatable conditions encountered by humans and animals. Wound repair is a well highly coordinated process that involves a series of overlapping phases, inflammation cell proliferation, matrix deposition and tissue remodeling, underlying repair is a complex dynamic series of events including clotting, inflammation, granulation tissue formation, epithelialization, neo-vascularization, collagen synthesis and wound contraction [Singer et al, 1999]. Briefly, the wound healing process consists of three major stages. First inflammatory cells from the surrounding tissue move towards the lesion site. Subsequently, fibroblasts appear and begin to produce collagen connective fibers that impart tensile strength to the regenerating tissue. Simultaneously, numerous capillaries begin to form to supply the site with nutrients and oxygen, while the epithelial cells at the edge of the wound start filing in the area under the scab. In the third and final
phase, the new epithelium forms and the wound considered healed (Wang et al., 2008).

Chitosan oligomers have also exhibited wound-healing properties, it is suggested that their wound-healing properties are due to their ability to stimulate fibroblast production by affecting the fibroblast growth factor. Subsequent collagen production further facilitates the formation of connective tissue (Howling et al., 2001).

2.10 Antitumor Activity

An antitumor activity of chitosan has been claimed by inhibition of the growth of tumor cells mainly due to an immune stimulation effect. However this property is very controversial (Kim and Rajapakse, 2005). Jeon and Kim (2002), have found that chitosan oligomers possess antitumor activities tested both in vitro and in vivo. Studies carried out using mice that had ingested low molecular weight chitosan revealed significant antimetastatic effects of chitosan against Lewis lung carcinoma. Partially deacetylated chitin as well as chitin with a carboxymethyl group have also been effective to demote tumor progression (Tsukada et al., 1990).

The suggested mechanism involves immunostimulating effects of chitin and its carboxymethyl derivatives via stimulation of cytolytic T-lymphocytes. This activity increases with smaller molecular sizes and it is suggested that they have
immunostimulating effects that activate peritoneal macrophages and stimulate non specific host resistance. However, higher molecular weight oligomers have also exhibited antitumor activity. The same mechanism has been suggested for their activity via increased production of lymphokines by activated lymphocytes (Suzuki et al, 1986).

Ueno et al (2001) studied the effect of chitosan on tumor growth and metastasis. The activation of macrophages by chitosan is suggested to mediate its antitumor effects in vivo, while its angiogenic inducing properties may be the harmful effects of chitosan, such as promotion of tumor growth and invasion.

2.11 Benzidine

Benzidine is a manufactured chemical that does not occur naturally. It is a crystalline (sandy or sugar-like) solid that may be grayish-yellow, white, or reddish-gray. It will evaporate slowly from water and soil. Its flammability, smell, and taste have not been described. Benzidine also has other names, such as 4,4'-diphenylenediamine or Fast Corinth Base B (a registered trade name). In the environment, benzidine is found in either its "free" state (as an organic base), or as a salt (for example, benzidine dihydrochloride or benzidine sulfate). In air, benzidine is found attached to suspended particles or as a vapor. In the past, industry used large amounts of benzidine to produce dyes for cloth, paper, and leather. However, it has not been made for sale in the United States since the mid-1970s. Major U.S. dye companies no longer make benzidine-based dyes.
Benzidine is no longer used in medical laboratories or in the rubber and plastics industries. However, small amounts of benzidine may still be manufactured or imported for scientific research in laboratories or for other specialized uses. Some benzidine-based dyes (or products dyed with them) may also still be brought into the United States (AGGIH, 1998).

2.11.1 Benzidine In Environment

In the past, benzidine entered the environment largely when it was being made or used to produce dyes. Industry released it to waterways in the form of liquids and sludges, and transported benzidine-containing solids to storage or waste sites. Benzidine was sometimes accidentally spilled, and it was released to the air as dust or fumes. For the most part, companies no longer make or use benzidine, and the government strictly regulates these activities. Today, most benzidine still entering the environment probably comes from waste sites where it had been disposed. Some may also come from the chemical or biological breakdown of benzidine-based dyes, or from other dyes where it may exist as an impurity. Only very small amounts of free benzidine will dissolve in water at moderate environmental temperatures. When released into waterways, it will sink and become part of the bottom sludge. Benzidine salts can dissolve more easily in water than free benzidine. Only a very small portion of dissolved benzidine will pass into the air. Benzidine exists in the air as very small particles or
as a vapor, which may be brought back to the earth's surface by rain or gravity. In soil, most benzidine is likely to be strongly attached to soil particles, so it does not easily pass into underground water. Benzidine can slowly be destroyed by certain other chemicals, light, and some microorganisms (for example, bacteria). Certain fish, snails, algae, and other forms of water life may take up and store very small amounts of benzidine, but accumulation in the food chain is unlikely (Adlercreutz H, 1995)

The general population is not likely to be exposed to benzidine through contaminated air, water, soil, or food. Benzidine is a manufactured chemical that does not occur naturally in the environment. Today, U.S. industry makes and uses very little (if any) benzidine, and no releases to air, water, or soil are reported on the Toxic Release Inventory (TRI). Only rarely has benzidine been detected in areas other than waste sites, and it has not been found in food. Some dyes used to color foods or drinks may contain impurities that can be broken down to benzidine once inside the body. If you live near a hazardous waste site, you could be exposed to benzidine by drinking contaminated water or by breathing or swallowing contaminated dust and soil. Benzidine can also enter the body by passing through the skin. Some quantities of dyes made from benzidine may still be brought into the United States. These may contain small amounts of benzidine as a contaminant, or chemicals that may be broken down in the body to benzidine. If you use such dyes to dye paper, cloth, leather, or other materials, you may be exposed by breathing or swallowing dust, or through skin contact
with dust. You may be exposed in a similar way if you work at or near hazardous waste sites (Aldrich, 1998)

### 2.11.2 Exposed To Benzidine

The general population is not likely to be exposed to benzidine through contaminated air, water, soil, or food. Benzidine is a manufactured chemical that does not occur naturally in the environment, some of the dyes used to color foods or drinks may contain impurities that can be broken down to benzidine once inside the body. Benzidine can also enter the body by passing through the skin when exposed to benzidine by drinking contaminated water or by breathing or swallowing contaminated dust and soil. Some quantities of dyes may contain small amounts of benzidine as a contaminant, or chemicals that may be broken down in the body to benzidine.

### 2.11.3 Health effects

**a) Cardiovascular Effects**

The reported oral LD50 is 214 mg/kg body weight for mice and 309 mg/kg body weight for rats (DOT 1972). No studies were located regarding cardiovascular effects in humans after oral exposure to benzidine. One study reported that benzidine administered orally to 6 rabbits (50 or 100 mg 1 day/week, or approximately 13 or 26 mg/kg/day), and 2 dogs (100 mg 1
day/week, or approximately 7.9 mg/kg/day) for 20–128 days induced myocardial atrophy and interstitial myocarditis (Oida 1958a, 1958b).

b) Hematological Effects

No studies were located regarding hematological effects in humans after oral exposure to benzidine. Hemosiderin pigment in the spleen was found in significant amounts for both sexes of the two crosses examined (Littlefield et al, 1984).

c) Hepatic Effects

No studies were located regarding hepatic effects in humans after oral exposure to benzidine. Cirrhosis of the liver was found in rabbits (perhaps also in dogs, but this was not clearly indicated) orally exposed to approximately 13 or 26 mg/kg/day of benzidine, 1 day/week over a period of 20–128 days (Oida 1958a, 1958b).

d) Renal Effects

No studies were located regarding renal effects in humans after oral exposure to benzidine. Nephrosis, nephritis, hematuria, and proteinuria were reported in rabbits orally exposed to approximately 13 or 26 mg/kg/day of benzidine 1 day/week over a period of 20–128 days (Oida 1958a, 1958b).
e) Immunological and Lymphoreticular Effects

No studies were located regarding immunological effects in humans after oral exposure to benzidine. Two animal studies have been reported that address the immunotoxicity of benzidine following oral exposure. In a study by Luster et al. (1992), an unspecified number of female B6C3F1 mice were exposed by gavage for 5 days to three unspecified doses of benzidine.

f) Reproductive Effects

No studies were located regarding reproductive effects in humans after oral exposure to benzidine. The only information regarding reproductive effects in animals is that from a lifetime study in mice, which reported a 31% incidence of atrophy of the ovaries in animals given benzidine in the drinking water at a dose level of approximately 7.2 mg/kg/day (80 ppm) compared to 11% in control mice (Littlefield et al. 1983).

g) Cancer

A retrospective study of residents who lived, during the 1970s near a Superfund site, that was contaminated with benzidine, β-naphthylamine, and benzene detected an excess of bladder cancer, leukemia, and other lymphomas, as well as cancers at several other sites (salivary gland, larynx, bone and jaw, uterus and chorion, rectum, and breast) (Budnick et al. 1984).
A number of animal studies indicate that oral exposure to benzidine can increase the incidence of a variety of tumors. In female rats given 3.4 mg/kg/day once every 3 days for 30 days, 5 out of 10 animals developed mammary carcinomas within 9 months, compared to 5 out of 132 in the controls (Griswold et al. 1968). In hamsters fed either 0.1% benzidine base (61 mg/kg/day) or 0.1% benzidine dihydrochloride (equivalent to 44 mg/kg/day benzidine base).

The exposure of benzidine and preventive measurement is regulated by environmental pollution Act (EPA) and food and drug administration Act (FDA).