Chapter One

Introduction and Literature Review

1.1: Introduction:

Cancer is a term used to describe all types of malignant tumors even if it was sarcoma or carcinoma (Anderson, 1980; Kuehnel 2003). It is considered as one of the most dangerous health problems in all communities especially in Iraq, due to the different weapons used in the wars during the past years (ICR 2004).

The bladder cancer is the second most common cancer of the genitourinary tract (Collado *et al,* 2000; Bryan *et al,* 2004). It is usually transitional cell carcinoma, and it spread locally by direct invasion. This disease is widely spreads in the industrial countries as well as in the schistosomiasis endemic countries (Edwards and Bouchier, 1991). People with bladder cancer usually treated either by surgery, irradiation, or chemotherapy (William *et al,* 1998; Unyime *et al,* 1998; Scher and Norton, 2006).

Development of drug resistance is a common problem in cancer chemotherapy, there is plenty information on how microorganisms develop resistance to various drugs, but the knowledge about the mechanisms underlying drug resistance in mammalian cell is still poor (Iqbal, 2003).

In this study the response to two anti- cancer drugs (6-Thioguanine, and Methotrexate) were scored in the lymphocytes obtained from bladder cancer patients by using certain cytogenetic

parameters (Blastogenic Index, Mitotic Index, Chromosomal Aberrations, and Micronucleus).

The 6- thioguanine (6- TG) is a chemotherapeutic drug, it is a cytostatic metabolite; it is effective as a Leukemia treatment agent as well as immunosuppressant (Aubrecht *et al*, 1997). The 6-TG is a purine analoge (guanine analoge). The antipurines can both inhibit nucleotide and nucleic acid synthesis and some times they can do both (Aubrecht *et al*, 1997). Resistance to 6- TG however, indicates a mutation at HPRT gene (Morley *et al*, 1983; Aubrecht *et al*, 1997).

The Methotrexate (MTX) was in used since the 1951 as chemotherapeutic agent (Osborn *et al*, 1958). It is a folate antagonist which kills the proliferating cells by inhibition of the activity of the dihydrofolate reductase enzyme (DHFR) (Jolivet *et al*, 1995). Resistance to MTX is indicated as a gene amplification of DHFR (Iqbal, 2003).

Development of drug resistance is one of the main problems in cancer treatment; it is a very serious problem that may lead to recurrence of disease or even death, therefore several studies have been made to overcome this problem.

1.2: Aim of the Study:

The aims of the study are:

1. To study the effects of the 6-TG, and MTX on cytogenetic parameters (BI, MI, and MN).

2- To asses the CA in the lymphocytes of BC patients treated with MTX and 6-TG *in vitro*.

1.3: Literature Review

1.3.1: Cancer:

Cancer is a disease of the cells which make up all the organs and tissues of the body (internet #1). In this disease the regulation of the cell cycle goes away and normal cell behavior is lost (Durran *et al*, 2001). Cancer cells show unlimited growth; they escape the rules of differentiation and grow wild. They also have the ability to infiltrate and destroy normal tissues; most malignant tumors are also capable of spreading to new sites by metastasis (Mary and Gospodarowicz, 2006). Cancer usually caused by mutations, mainly in somatic cells. Some individuals have inherited genetic mutations that predispose them to develop specific types of cancer (Anderson, 1980).

The wall of the bladder is lined with cells called transitional cells and sequamous cells (Mary and Gospodarowicz, 2006); more than (90 %) of bladder cancers are transitional cell carcinoma (TCC)

(Anderson, 1979; Gromova *et al*, 2002). About (6 – 8 %) of bladder cancers are sequamous cell carcinoma, and about (2 %) are adenocarcenoma (Burch *et al*, 2000). Sequamous cell carcinoma is a relatively rare tumor in the western world (Shaaban *et al*, 1997).

Bladder cancer was first described in 1895 (Walter and Israel, 1987). This disease is the second most common cancer of the genitourinary tract (Collado *et al*, 2000; Bryan *et al*, 2004). In Iraq it is the third most common malignancy (ICR; 2004), in Egypt is the most common malignancy (Mostafa *et al*, 1999).

It is the fourth most common malignancy in men and the eighth most common in women (Kaseem *et al*, 2002). It has the sixth highest incidence of all tumors in the developed world (Zou *et al;* 2000), the fourth most common cancer in the United States, the fifth most common malignancy in England and Wales (Bryan *et al*, 2004),

The basis for bladder cancer development and progression is complex and involves genetic abnormalities. These abnormalities yield phenotypic changes that allow normal cell to become cancerous and finally acquire the malignant phenotype (Jung *et al*, 2000).

1.3.2: Risk factors of bladder cancer:

Most cancers in adults arise spontaneously in response to unknown stimuli, but a few can be attributed with certainty to some tangible preceding cause.

The factors generally recognized as possible causes of human cancers are either chemical carcinogens, physical carcinogenic agents, viruses, hereditary predisposition, hormonal imbalance, and cancer may be occur due to chronic disease (Walters and Israel, 1987).

No one knows the exact cause of bladder cancer. However, it is clear that this disease is not contagious. People who get bladder cancer are more likely than other people to have certain risk factors. Still most people with known risk factors do not get bladder cancer, and many who do get the disease have non of these factors (Iqbal, 2003).

Risk Factors for Bladder Cancer are:

1.3.2.1: Biological Factors:

1.3.2.1.1: Schistosomiasis:

Schistosomiasis is a major health problem in many subtropical developing countries (Sheweita *et al*, 2003).

Schistosoma. haematobium cystitis appears to be related to the development of sequamous cell carcinoma (Jung *et al*, 2000). In

Egypt, it is the most common malignant tumor in individuals where shistosomiasis is endemic ,in Iraq there is decline in the incidence of sequamous cell carcinoma of the bladder, it is now second in incidence to transitional carcinoma (Rifat, 2000).

1.3.2.1.2: Hereditary factors:

Familial clustering of bladder cancer, especially of relatively young individuals, has provided support to the concept that there are may be a genetic component involved in some bladder cancers (Goldgar *et al*, 1994).

1.3.2.1.3: Gender, Race, and Age:

Men are approximately three times more likely than women to develop bladder cancer (Stamouli *et al*, 2004). Men also have traditionally increased exposure to putative carcinogene found in their work places and cigarette smoke (Jung *et al*, 2000).

Whites get bladder cancer twice as African, American, and Hispanics. The lowest rates are among Asian (Herr, 2006). People under forty rarely get this disease. About 80% of newly diagnosed TCCs in people who are over 60 years of age and over (Jung *et al*, 2000; Stamouli *et al*, 2004).

1.3.2.2: Chemicals:

1.3.2.2.1: Cigarette smoking:

Cigarette smoking is another risk factor for bladder cancer (Golka and Goebell, 2006). Investigations indicate that such risk

might be due to aromatic amines which are present in tobacco smoke (Vineis, 1992).

The aromatic amines are found not only in cigarette smoke but also in several industrial chemicals. One potential mechanism by which amines because carcinogenesis is by forming DNA adducts that resulted in transitional mutations (Kadlubar and Badawi, *1995; Cao et al, 2005).*

1.3.2.2.2: Occupational exposure:

Occupational exposure to chemicals has been identified as the second most important risk factor for blabber cancer after smoking (Ji *et al*, 2005). The exposures to chemicals used in dye, rubber, and textile manufacturing have been estimated to be responsible for up to 20 % of bladder cancer cases (Kiemeney *et al*, 1996).

Most of these chemicals are aromatic amines such as benzidine, and beta- naphthylamine and certain azo dye. They take several years to accumulate and thus account for the long latent period before the development of bladder cancer (Golka and Goebell, 2006).

1.3.2.2.3: Arsenic:

Humans are environmentally exposed to arsenic primarily through drinking water. Ingestion of arsenic in drinking water is a strong risk factor for several forms of cancer including bladder cancer (Moore *et al*, 2002).

It is very difficult to study how arsenic might cause cancer. For unknown reasons, arsenic does not cause cancer in laboratory animals, Cell culture studies have shown that arsenic can break chromosomes, stop cell division, and inhibit DNA repair, among other effects (Hei, 2002).

1.3.2.5: Cyclophosphamide:

Medical treatment with cyclophosphamide which is a drug used in cancer chemotherapy increase the risk of getting bladder cancer nine folds (Tuttle *et al, 1988*).

1.3.2.3: Other factors:

1.3.2.1: Personal history of bladder cancer:

People who have had bladder cancer have an increased risk of getting the disease again (Jung *et al*, 2000).

1.3.3: Diagnosis of bladder cancer:

Several tests have been used for diagnosis of bladder cancer, these are:

A- Urine test: check the urine for blood, cancer cells, and other signs of the disease.

B- Intravenous pyelogram: in this test a contrast agent (radio paque dye) is administered though a vein (intravenously) and X- ray are taken as the dye moves through the urinary tract.

C- Cytoscope and biopsy: The doctors use a thin, light tube (cytoscope) to look directly into the bladder. The doctor inserts the cystoscpe into the bladder through the urether.

In biopsy, tissue samples are taken from the lesions and examined for cancer cells (Guinan and Rubenstein, 2006).

1.3.4: Stages of bladder cancer:

The bladder cancers are classified into several stages, according to the degree of spread of disease (Mary and Gospodarowicz, 2006), those stages are:

-Stage 0: the cancer cells are found only on the surface of the inner linning of the bladder. It is also known as super facial cancer. About 80 % of patients initially present with superficial tumor (Collado *et al*; 2000).

-Stage 1: the cancer cells are found deep in the inner lining of the bladder, they have not spread to muscle of the bladder.

-Stage 2: the cancer cells have spread to the muscles of the bladder.

-Stage 3: the cancer cells have spread through the muscular wall of the bladder to the layer of tissues surrounding of the bladder.

-Stage 4: the cancer extends to the wall of abdomen or to the wall of the pelvis. The cancer cell might spread to lymph nodes and other parts of the body far away from the bladder, such as the lungs.

1.3.5: Treatment of bladder cancer:

People carry out bladder cancer can be cured by surgery, irradiation, or combination of modalities that include chemotherapy.

1.3.5.1: Surgery: When cancer involves the pelvic or has spread to other parts of the body, the surgeon may suggest Surgery to remove cancer (Scher and Norton *et al*, 2006).

Transurethral resection (TUR) is a surgical procedure that is used both to diagnose bladder cancer and to remove cancerous tissue from the bladder.

Surgery to remove bladder (Cystectomy) is the surgical removal of all (radical) or part (partial) of the bladder. It is used to treat bladder cancer that has spread into the bladder wall (stages II and III) as either a first occurrence or as a cancer that returns (recurs) following initial treatment.

A radical cystectomy removes the whole bladder as well as the surrounding pelvic organs (Howard *et al*, 2006).

1.3.5.2: Chemotherapy: Means using of drugs to stop the growth of cancer cells. There are many kinds of chemotherapeutic drugs which are used for the treatment of bladder; the following chemotherapeutic agents may be given to treat bladder cancer:

- Cisplatin
- Doxorubicin
- Cyclophosphamide
- Adriamycin
- Methotrexate
- Mitoxantrone
- Vincristine
- Taxol
- Thiotepa
- Prednisone
- Mitomycin C
- Epirubicin

(William *et al*, 1998; Scher and Norton, 2006)

Chemotherapy may be given in different ways:

-Orally or intramuscularly, or by catheter into the bladder (intravesical chemotherapy). Some of these drugs are given in cycles so that treatment periods alternate with rest periods. Chemotherapy can kill cancer cells both inside and outside the bladder area (William *et al*, 1998).

1.3.5. 2.1: Medications through a vein (IV)

A. Doxorubicin is an anthracycline antibiotic medication. Epirubicin and valrubicin are also anthracycline antibiotics that may be used.

B. M-VAC is a combination of methotrexate, vinblastine, doxorubicin, and cisplatin. Methotrexate slows or stops the growth of cancer cells in the body and it is frequently used in combination with other chemotherapeutic medications. Cisplatin is a heavy metal that causes cell death by interfering with the multiplication of cancer cells (William *et al*, 1998).

C. Gemcitabine is an antitumor medication that interferes with how cells divide and stops the growth of the cancer cells.

D. Paclitaxel or carboplatin are antitumor medications that slow or stop the growth of cancer cells in the body.

1.3.5.2.2: Medications through a catheter into the bladder

A. Mitomycin C is an antitumor antibiotic that interferes with the multiplication of cancer cells. When administered directly into the bladder, mitomycin may help prevent the recurrence of bladder cancer (William *et al*, 1998; Howard *et al*, 2006)

B. Bacillus Calmette-Guerin (BCG) may stimulate an immune response or inflammation in the bladder wall to destroy cancer cells within the bladder. This is known as biological therapy (William *et al*, 1998; Scher and Norton *et al*, 2006)

1.3.5.3: Radiation therapy:

Also known as radio therapy, which is the use of high energy rays to kill cancer cells. There are two types of radiation therapy:

A. External Radiation: a large machine out side the body aims radiation at the tumor area (Internet #1).

B. Internal Radiation: a small container of radio active substance is placed into the bladder through the urethra. The patients stay in the hospital for several days. Once the implant is removed, no radio activity is left in the body (Internet #1).

1.3.5.4: Biological therapy:

Also called immunotherapy, uses of the body's natural ability (immune system) to fight cancer, the most common immunotherapy is BCG, *Bacillus Calmette – Guerin* (BCG) has been in use since 1980's, and it is the most proven and effective form of immunotherapy at this point of time (Unyime *et al*, 1998).

BCG is an attenuated strain of *Bacillus Calmette – Guerin*, when used intravascular for treatment of bladder cancer, it is though to cause a local chronic inflammatory response involving macrophage and leukocytes infiltration of the bladder. By a mechanism not fully understood, this local inflammatory response leads to destruction of super facial tumor cells of the urothelium (Lamm *et al*, 1991).

1.3.6: Bladder cancer cytogenetics:

The development of cancer is associated with a fundamental genetic change within the cell and there is overwhelming evidence that mutations can cause cancer- mutation defined as a change in the genome- (Kumar and Clark, 2000).

Cancer genes can be classified in to three major categories, those are the tumor suppressor, oncogenes, and those participate in DNA repair gene (Jorde *et al*, 2000).

Methods such as interphase multicolor fluorescence in situ hybridization (FISH), competitive genomic hybridization (CGH), and micro array technologies have been used to detect cytogenetic changes of cancer cells (Ashman *et al*, 2002; Bezrookove *et al*, 2003).

Cytogenetic analyses of bladder cancer have shown several nonrandom aberrations; those may include both chromosomes and genes aberrations (Ghali *et al*, 2000; Stamouli *et al*, 2004).

1.3.6.1: Chromosome 9:

Changes involving chromosome 9 have been reported as the sole cytogentic aberration in some bladder cancer cases (Stamouli *et al*, 2003).

The chromosome 9q allelic losses have been reported as a frequent and early event occurring in bladder cancer (Orlow *et al*, 1994). Age, gender, and smoking were not significantly associated with chromosome 9q allele loss (Hirao *et al*, 2005).

It has been postulated that a candidate tumor suppressor gene may reside on this chromosome, alteration of which may lead to the development of bladder cancer (Orlow *et al*, 1994; Stamouli *et al*, 2004; Hiro *et al*, 2005).

The losses of chromosome 9 alleles occur in superficial as well as invasive bladder cancer (Miyao *et al*, 1993; Stamouli *et al*, 2004).

1.3.6.2:Chromosome 7:

Studies showed that, increased copies for chromosome 7 with numerical chromosomal aberrations are highly related to bladder cancer, Trisomy (Stamouli *et al*, 2004) or tetrasomy (Waldman *et al*, 1991) is a frequent event in bladder cancer, and some times described as the sole anomaly. More often, it coexists with other complex aberrations and could be associated with an aggressive tumor behavior (Waldman *et al*, 1991; Stamouli *et al*, 2004).

1.3.6.3: P53:

The *P53* gene which is located on chromosome 17 encodes for a transcription factor (Jung *et al*, 2000). Loss of hetrozygosity on chromosome 17 is very common aberration in bladder cancer (Tasi *et al*, 1990)

In the normal cells, *P53* defends against uncontrolled proliferation by causing G1 cell cycle arrest and apoptosis in response to DNA damage by radiation or mutagenic chemicals (Cordon, 2004; Bitton *et al*, 2005).

Mutations in the *P53* tumor suppressor gene are the most common somatic mutations identified among cancers; about 50 % of cancer cases include mutations in the P53 gene (Schroeder *et al*, 2003).

Those mutations in the *P53* gene might occur due to exposure to specific carcinogens such as UV light, or may occur spontaneously (Schroeder *et al*, 2003). Alterations in *P53* could assist in identifying patients presenting with high risk superficial tumors (Cordon, 2004). *P53* alteration is a predictor of decreased survival (Jung *et al*, 2000).

1.3.6.4: Retinoblastoma gene (RB):

The RB gene is located on chromosome 13 (Erbesdobler *et al*, 1998). It is the critical regulator of cell growth (Cordon, 2004). Studies have shown that, there is an altered pattern of expression or mutations in RB (Cordon, 2004). Altered RB expression occurs in all grade and stages of bladder cancer, but it is more commonly associated with invasive tumors (Presti *et al*, 1991).

1.3.6.5: Activation of H- ras oncogene:

Oncogenes may be contributed to transformation and progression by being either over expressed or mutated to produce onco protein (Jung *et al*, 2000).

One of the most important mechanisms by which oncogene are over expressed in bladder cancer is through gene amplification (CLi *et al*, 2005).

H- ras oncogene is located on chromosome 11 (Burchill *et al*, 1994). A simple point mutation in the sequence of the ras gene, in which the guanine is converted to cytocine is frequently associated with bladder cancer (Eric *et al*, 2006). This point mutation will lead to over expression of H- ras gene. Mutations in the H-ras gene are considered as an early event in the development and progression of a significant proportion of human bladder cancer cases (Burchill *et al*, 1994).

Table (1- 1)

Genetic changes in bladder cancer patients

	Changes at Loci or Chromosome	Reference
1	9q allelic losses	Orlow *et al*,1994
2	Mutations in the P53 tumor suppressor gene	Schroeder *et al*, 2003
3	Altered RB expression	Presti *et al*, 1991

4	Over expression of H- ras	Burchill et al, 1994
5	Increased copies for chromosome 7	Stamouli *et al*, 2004,Waldman *et al*, 1991
6	loss of hetrozygosity on chromosome 17	Tasi *et al*, 1990

1.4: Drug resistance:

One of the main causes of failure in the treatment of cancer is the development of drug resistance by the cancer cells. This is a very serious problem that may lead to recurrence of disease or even death. While it is thought that the majority of cancers arise from a single precursor cell, it would be an error to view a tumor as consisting of a collection of genetically identical cells. One of the hallmarks of cancer is an increase in genetic instability and mutation rates. These changes mean that dividing cancer cells acquire genetic changes at high rates. Practically, this means that the cells in a tumor, while similar, are not identical, when exposed to a cancer drug. Those cells that are sensitive to the effects of the drug are killed. Those that are resistant will survive and multiply, the result is the re- growth of a tumor that is not sensitive to the original drug (Internet # 2).

Some of the major mechanisms of drug resistance identified in mammalian cells include:

- Altered transport of the drug.
- Increase in total amount of target enzyme/ protein.
- Alteration in target enzyme/ protein.
- Elevation of cellular glutathione.
- Inhibition of drug induced apoptosis (Berendsen *et al*, 1997; Bush *et al*, 2002; Iqbal, 2003).

1.4.1: Altered transport of the drug:

When there is a change in one of the transport proteins of a particular protein, then the influx of the drug in cancer cell or its efflux might get affected, resulting into decreased quantity of the drug inside the cancer cell (Moscow *et al*, 1995)

Decreased influx of the drug may occur due to mutation in surface membrane protein that is involved in the transport of the drug inside the cell or it's decreased expression might lead to reduced uptake of the drug, and, hence, the process inside the cell will not be inhibited (Wong *et al*, 1995)

Increased efflux of the drug from cancer cell might occur due to increase expression of p- glycoprotein, which leads to multi- drug resistance (MDR) which is a complex phenotype whose predominant feature is resistance to a wide range of structurally

unrelated cytotoxic agents, many of which are anti cancer drugs. Over expression of p – glycoprotein which a plasma membrane glycoprotein will lead to increase efflux of the drug and so leads to drug resistance (Dietel, 1996; Claudio *et al*, 1996; Bleicher *et al*, 1999; Kolfschoten *et al*, 2000; Kruh *et al*, 2001).

Also decreased poly glutamate formation inside the cell may lead to drug efflux, because of reduced level of ploy- glutamate possibly because mutation in its gene, would lead to decreased poly glutamation inside the cell and hence, rapid efflux of drug (Wong *et al*, 1995).

1.4.2: Increased in total amount of target enzyme/protein (gene amplification):

The increase in the total amount of the target enzyme/ protein is occurring due to gene amplification.

As an alternative to change in the enzyme itself, the amount of the enzyme may be increased. The cause of this increase is an amplification of the number of the structural genes of the enzyme.

The amplification of the gene is stable if it is localized in a specific region of a chromosome, or unstable if localized in the nucleus as extra chromosomal DNA (Lewin, 1998; Kundig *et al*, 1998).

1.4.3: Alteration in the target enzyme/protein:

Presumably because mutation, there is a structural change in the target enzyme such that the normal high affinity for the drug is lost. When this happens, the enzyme would no longer be inhibited by the drug at least at the conventional doses (Riordan *et al*, 1985).

1.4. 4: Elevation of cellular glutathione:

Glutathione is a tri-peptide (L-g-glutamyl-L-cysteinyl-glycin) present virtually in all mammalian cells. It offers protection to cells by the destruction of reactive oxygen compounds, free radicals, and other toxic compounds of endogenous and exogenous origin. Because property it has an important role in drug detoxification (Ozole *et al* 1990).

Drug resistant in tumor cells have been shown to contain levels of GSH several orders of magnitude higher than those measured in wild type cells (Lee *et al*; 1996, Berendsen *et al*, 1997).

GSH may reduce cytotoxicity by facilitating of metabolism of drugs to less active compounds or by detoxification of the free radical (Meister, 1983; Iqbal, 2003).

1.4. 5: Inhibition of drug induced apoptosis:

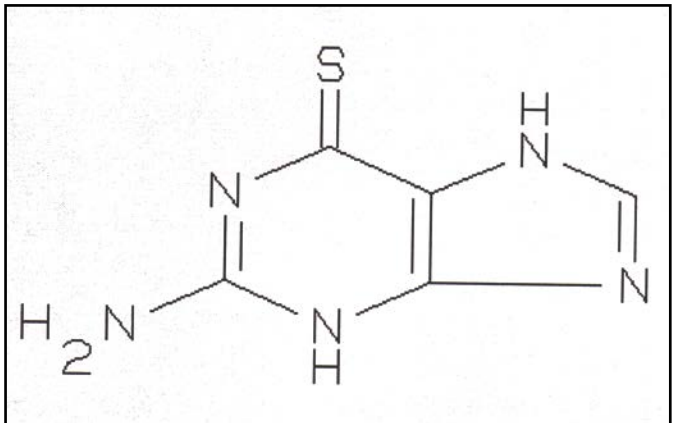
Killing of cancer cells by various cytotoixic approaches such as anticancer drugs, radiation, or immunotherapy, is predominantly mediated through induction of apoptosis in target cells.

Thus, defects in apoptosis programs that suppress cell death can also confer drug resistance. Since apoptosis is a gene directed program, it can be distributed by genetic mutations, mutations often results in an increased growth rates or a block in apoptosis , leading to tumor initiation, progression and resistance to current treatment approached (Fulda *et al,* 2001).

Mutations in *P53* gene or its deletion by cytotoxis drugs could lead to defective apoptotic pathway resulting into drug resistance tumors (Fisher, 1994; Gorlick *et al*, 1996). Mutations in the *P53* tumor suppressor gene lead to uncontrolled cell division and are found in over 50 % of all human tumors. In normal cells, *P53* defends against uncontrolled proliferation by causing G1 cell cycle arrest and apoptosis (cell suicide) in response to DNA damage by radiation or mutagenic chemicals.

P53 mutations contribute to tumor formation as they contribute to uncontrolled cell division regardless of DNA damage (Asaf, *et al*, 2005).

1.5: 6- Thioguanine (6TG):



Chemical structure of 6-Thioguanine

(Internet # 3)

6-TG is a cytostatic metabolite; it is effective as Leukemia treatment agent as well as immunosuppressant (Aubrecht et *al*, 1997).

The 6-TG is a purine analoge (guanine analoge), the antipurines can both inhibit nucleotide and nucleic acid synthesis and some times they can do both (Internet # 8). 6-TG is first convert to 6 TGMP by Hprt in purine salvage pathway. First the 6 TGMP works as a pseudo feed back inhibitor of glutamine -5-phosphoribosylpyrophosphate amidotransferase, and block purine biosynthesis. Second, 6 -TGMP inhibits IMP dehydrogenase and thus purine interconversion.

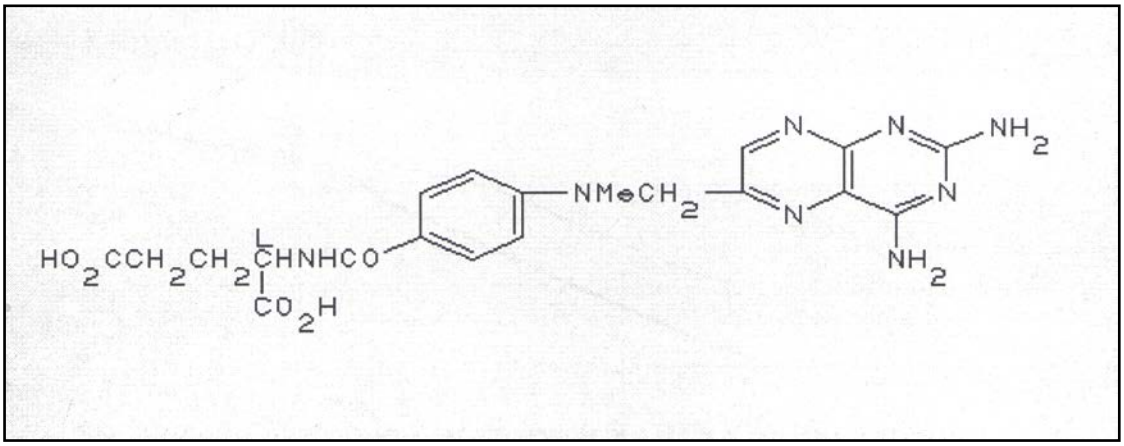
The net consequence of this activity is block of the synthesis and utilization of purine nucleotides. Third, 6TGMP, after conversion to the triphosphate form is incorporated into either DNA or RNA (Aubrecht *et al*, 1997).

Resistances to 6- TG usually occur due to mutations of the gene for hypoxanthine - guanine - ribosyl transferase (HGPRT) (Morley *et al*, 1983; Aubrecht *et al*, 1997).

Increased level of alkaline phosphatase which increase the break down of the nucleotide form consider as one mechanism for 6-TG resistant (Beverly *et al*, 1981).

Usually patients receiving chemotherapy or radiation therapy showed significantly higher numbers of 6-TG resistant lymphocytes than healthy controls (Beverly *et al*, 1981).

1.6: Methotrexate (MTX):



Chemical Structure of Methotrexate

(Internet # 3)

Methotrexate is classified as an anti – metabolite drugs, it is a folate antagonist, whose mode of action in not fully established. Almost 50 years ago, methotrexate (MTX) was developed as a chemotherapeutic agent for the treatment of cancer, especially leukemia. Subsequently, MTX was found to play a major therapeutic role in non- neoplastic disease, acting as an anti –

inflammatory and immuno- suppressive drug (Majumodor and Aggarwal, 2001).

How MTX mediates its effects at the cellular level is not fully under- stood. MTX is taken up by cells and tissues and converted to MTX- polyglutamtes, long – lived derivatives that retain biochemical and biological activity within the cell. MTX – polyglutamates can competitively inhibit dihydrofolate reductase (DHFR), which ultimately affects both purine and pyrimidine nucleotide biosynthesis. Thus during the treatment of cancer with MTX, the malignant cells become starved for purine and pyrimidines, precursors of DNA and RNA required for proliferation (Majumodor and Aggarwal, 2001; Serra *et al*, 2004).

Resistance to MTX may occur due to certain mechanisms, these mechanisms include increase in DHFR enzyme level (due to DHFR gene amplification), or due to the impaired intracellular transport of MTX (Kundig *et al*, 1998; Serra *et al*, 2004).

1.7: Cytogenetic analysis:

Numerous diverse environmental and industrial chemicals are capable of causing cytogenetic changes in experimental animals. The potential for similar effects in man is obvious. The cytogenetic analysis is widely used system for the determination of the mutations caused by physical (radiation), chemical, (environment) or biological (virus infection) agents.

The Cytogenetic analysis is methods that permit direct image analysis for the chromosomes and genetic material damage (Carrano, 1985).

The classical method of assessing cytogenetic damage is the examination of metaphase preparation of cells treated with test agent either *in- vivo* or *in- vitro* (Heddle *et al*, 1977).

The *in-vivo* system carry out by using test animals such as mouse (Grawe *et al*, 1998); chicken embryos (Shubber *et al*, 2003), rabbits (Sokka *et al*, 2003).

The *in- vitro* systems usually carried out by culturing a certain kind of cells in suitable media and treat those cells with specific agent this system will guarantee the delivery of the agent to the cultured cells (Schneider and Lewis, 1982).

There are several parameters have been used in cytogenetic analysis including the mitotic index (MI), blastogenic index (BI), and chromosomal aberrations (CA) (Shubber and Al-Allak, 1986).

1.7.1: Mitotic index (MI)

The living cells are going in an ordered set of events, culminating in cell growth and division into daughter cells, these events known as mitotic cell division, in which the genetic materials is duplicated and divided into two daughter cells, the molecular mechanisms underlying the cell cycle are highly conserved in all organisms with nucleus eukaryotes (King *et al*, 1982).

The mitotic index (MI) is the number of mitosis in 1000 cells

MI= {Number of dividing cells/ (Number of dividing cells+ Number of non- Dividing Cells)} x 100.

(Stich and San, 1981).

1.7.2: Blastogenic index (BI):

Blastogenic index is a parameter used to determine the cellular response to certain mitogen which causes induction of proliferation (Soren, 1973).

Phytohemagglutinin (PHA) is a mitogen used with the Minimal Essential Medium or with the RPMI- 1640 Medium for the initiation of the lymphocyte *in vitro* to be stimulated to lymphoblast. PHA is known to stimulate the cellular mitosis. The stimulated lymphocytes are widely used to detect chromosome damaging agents (Morimoto and Wolff, 1980).

1.7.3: Chromosome aberration (CA):

The chromosomal aberration (CA) reflects an abnormality in the structure or number of the chromosomes. The CA may be induced by various physical, chemical, or biological agents (Kumar and Clark, 2000).

The abnormal chromosome number usually occur due to the failure of a chromosome or chromatid to separate either in mitosis or meiosis, one of the daughter cell will receive two copies of the chromosome, and the other will receive no copy of the chromosome, this will lead to polyploidy, trisomy, and monosomy (Mueller 1992; Kumar and Clark, 2000).

The structural aberrations are common in nature and occur spontaneously, but their frequency may increase due to the

exposure to radiation and other chemical mutagens (Evans and Oriordan, 1977).

There is evidence that increased frequency of CA in peripheral blood lymphocyte is predictor of cancer (Rossner *et al*, 2005).

1.7.3.1: Breaks: DNA breaks may occur due to exposure to various environmental factors such as radiation, chemicals, or viruses DNA breaks may occur in only one strand of the chromosome (Chromatide break) or double strand breaks (chromosome break) (Bosco and Haber, 1998; Rossner *et al*, 2005).

Chromosome breaks is one of the dangerous form of DNA damage that can cause alterations of structure and expression of important genes (Kumar and Clark, 2000).

Chromosome breaks are common in patients with certain diseases such as the mental retardation syndrome patients (Yip *et al*, 1996).

1.7.3.2: Deletion:

Refers to the loss of a segment of a chromosome. The deletion may be terminal or it can be interstitial deletion of a gene will removes its product in the growth pathway.

Usually the deletion is associated with the loss of tumor suppressor gene in cancer (Rabbits, 1994).

1.7.3.3: Ring chromosome:

Ring chromosome may occur when one or more chromosomes break, and during the repair process, the broken ends are rejoined incorrectly.

The ring chromosome may form in one of two ways, breaks in chromosome arms, and fusion of the proximal broken ends, leading to loss of distal material. Ring may also formed by telomere dysfunction triggering fusion of reactive chromosome ends without loss of genetic materials (Gisselsson, 2001).

1.7.3.4: Dicentric:

The chromosome may contain two centromeric regions, the two sister chromatid is attached to each other by two centromeres.

The dicentric chromosome can not pass cell division (Wang *et al*, 1993; Tucker *et al*, 2005).

1.7.3.5 Acentric chromosome:

The chromosome may loss its centromer, and the two sister chromatide of the same chromosome are not attached to each other (Tucker *et al*, 2005).

An acentric chromosome is usually lost during mitosis (Obe and Natarajan, 2004).

1.7.4: Micronucleus (MN):

Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosomes lagging at anaphase or from acentric chromosomal fragments.

Cytogenetic methods have used longest and most extensively for the biological monitoring of population exposed to physical and chemical carcinogens and mutagens (Sarto *et al*, 1989).

The micronucleus test for genotoxic damage has been used in many monitoring studies (Zetterberg, 1997).

The score of MN is faster and simpler that the scoring of CA (Grawe *et al*, 1998).

MN experiments using man, rats, and also wild- living rodent species, have met a lesser degree of success compared to experiments with mice (Zetterberg, 1997).

The MN technique has proposed as a method for measurement of chromosomal damage, MN require one cell division o be expressed and consequently, the conventional MN technique is very imprecise since the cells which have undergone only one division (Fenech 2000).

The yield of MN in human peripheral blood lymphocyte could be used as a biological dosimeter in cases of radiation exposure (Balasem *et al*, 1990).

It was found that all chromatide, chromosome, and isochromaid breaks, as well as a symmetrical and incomplete symmetrical exchange, will give rise to a centric fragments at mitosis, and that

these fragments are frequently excluded from the daughter nuclei and appear in the following interphase as micronucleus (Heddle *et al*, 1983).

Chapter Two

Materials and Methods

2.1: General laboratory equipments and apparatus:

The following equipments and apparatus were used through out the study:

Table (2 -1)

General Laboratory Equipments and Apparatus

Apparatus	Company
Autoclave	Gallenkamp (England)
Balance	Ohans (France)
Centrifuge	Gallenkamp (England)
Cold Incubator	Gallenkamp (England)
Distillator	Gallenkamp (England)
Electric Balance	Melter (Switzerland)
Electric Oven	Gallenkamp (England)
Electric Shaker	Merk (Germany)
Freezer	Ishtar (Iraq)
Laminar Air Flow	metalab (France)
Micropipette	Gelson (France)
Microscope	Olympus (Japan)
PH-Meter	Orien Research (USA)
Refrigerator	Ishtar (Iraq)
Water Bath	Gallenkamp (England)

2.2: Chemical Materials:

The following chemical materials were used in this study:

Table (2- 2)

The Chemical Materials

Materials	Company
RPMI- 1640	Sigma
Phytohaeaglutinine	Ridiobiology center of the Ministry of Science and Technology
Glycerin	Fluka
Giemsa Stain	Fisher
Glacila Acetic Acid	Fluka
Fetal Calf Serum (FCS)	Sigma
KCl (KH_2PO_4)	Merk
Absolute Methanol	Fluka
NaCl	Sigma
Na_2HPO_4	Sigma
KH_2PO_4	Sigma
HCl	Fluka
Heparine	Sigma
Penicillin	Sigma
Streptomycin	Sigma
Methotrexate	Hexal
6 - Thioguanine	Sigma
Hepes	Sigma

Colchicin	Houde

2.3: Preparation of Solutions:

2.3.1: Antibiotics:

Streptomycin was prepared by dissolving 1 g of streptomycin in 100 ml D.W.

Penicillin was prepared by dissolving 1.000.000 unit/100 ml D.W. Both antibiotics were sterilized by filtration under aseptic conditions.

Both antibiotics were stored at (-20 °C) until used (Shubber *et al*, 2000)

2.3.2: Phosphate Buffered Saline (PBS):

PBS was prepared by dissolving the following materials in one liter of D.D.W., the pH of the PBS was adjusted to (7.2), the prepared PBS were sterilized by autoclaving and stored at (4 °C) until use (Verma and Babu ,1989).

Compound	Amount
Sodium Chloride (NaCl)	8 gm
Potassium Chloride (KCl)	0.2 gm
Sodium Phosphate hydrate($Na_2PO_4.H_2O$)	1.15 gm
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 gm

2.3.3: Sodium Bicarbonate:

- 4.4 gm of Sodium Bicarbonate ($NaHCO_3$) was dissolved in 100 ml DDW, the prepared solvent kept in at (-20 C) until used.
- 0.5 gm of Sodium Bicarbonate ($NaHCO_3$) was dissolved in (100 ml) of D.W. and kept at (4° C) until it was used for Giemsa working solution (Allen *et al*, 1977).

2.3.4: Colchicine (Houde) :

One tablet of colchicine (0.5 mg) was dissolved in (10 ml) D.D.W. (stock). The working solution of colchicines was prepared by taking one ml of the previous stock and dissolved in (9 ml) of D.D.W., the working solution was kept frozen at(-20° C) until used.

2.3.5: Hypotonic Solution:

2.3.5. A: (0.075 M) KCl was prepared by dissolving (5.75 gm) of KCL in (1000 ml) D.W. The KCl was kept at the room temperature until used.

2.3.5. B: (0.1 M) KCL was prepared by dissolving (7.45 gm) KCl in (1000 ml) D.W. The KCl was kept at the room temperature until used.

2.3.6: Carnoy's Fixative:

The fixative was freshly prepared by adding one volume of Glacial acetic acid to three volumes of Absolute Methanol.

3 Methanols: 1 Acetic Acid (V/V)

(Shubber *et al*, 2003).

2.3.7: Normal Saline:

The normal saline was prepared by dissolving 8.5 g of NaCl in 1000 ml D.W., then sterilized and stored at (- 20° C) until used.

2.3.8: Heparin Solution:

The heparin was prepared by mixing (1 ml) of heparin with (24 ml) of sterile D.D.W. and distributed in sterile tubes, the tubes were kept at (4 °C) until used (Al- Amiry, 1999).

2.3.9: Phytohemagglutinin (PHA):

Phytohemagglutinin was obtained as frozen solution from Radiobiology Center of the Ministry of Science and Technology (Iraq), (1 ml) of the PHA was mixed with (19 ml) of normal saline (0.085 M), then sterilized by filtration using (0.22 µm) size filter, then it was stored at (-20 °C) until used.

2.3.10: Culture medium RPMI- 1640:

The Culturing media was prepared as follow:

Compound	Amount
RPMI 1640	10 gm
FCS (Heat inactivated)	100 ml
Hepes	3 gm
Sodium Bicarbonate (4.4 %)	15 ml
Penicillin	5 ml
Streptomycin	5 ml

The volume was completed with D.W. to 1000 ml, and the pH was adjusted to (7.2), then sterilized by filtration using (0.22 µm) size filter.

Then (2.5 ml) of the prepared medium was transferred into sterile tubes and kept at -20 °C until used (Shubber *et al*, 1991).

2.3.11: Methotrexate Solution (MTX) (Hexal):

The first concentration of (0.5 µg/٠,١ ml) was prepared by taking (0.04) ml of vial Methotrexate drug with a concentration of (25 mg / ml) was diluted under aseptic conditions with (200 ml) D.D.W., then the solution was sterilized by using Naljen filter paper (0.22) size filter, the prepared concentration was kept in refrigerator. (0.1 ml) of the prepared solution was added to the culture medium in order to obtain final concentration of (12.5 µg/ ml)

The second concentration (4 µg/0.1 ml) was prepared by dissolving (0.16 ml) of vial methotrexate with a concentration of (25 mg /1 ml), in (100 ml) D.D.W. under aseptic conditions. The solution was sterilized then by filtration with (0.22 µm) size filter, the

prepared solution was kept in the refrigerator. (0.1 ml) of the prepared concentration was added to the culture medium in order to obtain a final concentration of (100 µg/ml).

2.3.12: 6- Thioguanin (6 - TG):

The first concentration 0.2 µg/0.1 ml, this concentration was prepared by dissolving (0.0002) mg 6- TG in (100) ml D.D.W. under aseptic conditions. Then it was sterilized by filtration with (0.22) size filter. The solution was kept in the refrigerator. (0.1 ml) 0f the prepared solution was added to the culture media in order to obtain a final concentration of (50 µg/ml).

The second concentration (0.4 µg/0.1 ml) was prepared by dissolving (0.0004 mg) 6-TG in (100 ml) D.D.W. under aseptic conditions. The solution was then sterilized by filtration using (0.22 Um) size filters, the prepared solution kept in the refrigerator. (0.1 ml) of the prepared solution was added to the culture medium in order to obtain a final concentration of (100 µg/ml).

The third concentration (0.8 µg/ 0.1 ml) was prepared by dissolving (0.0008 gm) 6-TG in (100 ml) D.D.W. under aseptic conditions. The solution was sterilized by filtration using (0.22 µm) size filter. The prepared solution was kept in the refrigerator. (0.1 ml) of the prepared solution was added to the culture media to obtain a final concentration of (200 µg/ml).

The fourth concentration (1 µg/ 0.1 ml) was prepared by dissolving (0.001 mg) of 6-TG in (100 ml) D.D.W under aseptic conditions. The prepared solution was sterilized by filtration with (0.22 µm) size filter. The solution was kept in the refrigerator. (0.1 ml) of the prepared solution was added to the culture medium to obtain a final concentration of (250 µg/ ml).

2.3.13: Giemsa Stain:

The stock of Giemsa stain was prepared by dissolving (1 gm) of Giemsa powder in (33 ml) glycerin; the mixture kept in water bath at (60° C) for one hour, with continues shaking.

Then (66 ml) of absolute methanol was added to the previous mixture with continues shaking. The solution kept in the room temperature in a dark container at (37 °C).

The working solution was prepared by taking:

Compound	Amount
Giemsa Stain Stock	1 ml
Absolute Methanol	1.25 ml
Sodium Bicarbonate Solution	0.5 ml
Distal Water	40 ml

The compositions of Giemsa Stain

2.4: Subjects:

A blood sample of 56 subjects was included in this study. The subjects were divided in two groups. The first group includes 18 apparently healthy individuals (9 males and 9 females), which were considered as a control group.

The second group includes 40 patients with a clinical diagnosis of bladder cancer, only 18 patients (15 male, 3 females) give results, the rest (22 patients) fail to give results due to different reasons.

The patients were diagnosed by Dr. Hussein Lafta / Al Yarmouk Hospital. The age of the patients ranged from (31- 80) years, the age of the control ranged from (14- 59) years.

2.5: Blood Samples:

From each subject, (2.5 ml) were obtained under aseptic conditions; peripheral blood was used, which was obtained by vein-puncture using disposable syringe procoated with heparin.

The blood samples were collected during the period from December 2004- April 2006. The blood was placed in a cool- box and transferred to the laboratory.

The samples were used for cytogenetic analysis and micronucleus assay.

2.6: Experimental Design:

2.6.1: Standardizations:

- Standardization of colchicine:

In order to select the effective dose of colchicines which can give the best metaphase with good number and shape of chromosomes, the colchicine was prepared as described in section (2.2.4), three different volumes were taken (0.1 ml, 0.2 ml, 0.3 ml) and were used in the harvesting of cultured cells at different times (2 hr., 1 hr., and 1/2 hr.) in order to take the optimal concentration of colchicine with the optimal time.

The standardization was done by the use of blood collected from healthy individuals. It was found that the best doses were (0.2 ml) for (2 hr.), and (0.1 ml) for (1hr.).

Second experiment had been established to compare between the two doses and time by the measuring of BI, and MI.
The results showed that the best concentration was (0.2 ml) for (2 hr.

- Standardization of PHA:

To determine the suitable amount of PHA that can induce the blastogenesis, mitosis, or both, two concentrations of PHA were used to determine the best concentration.

The test was done by the use of blood collected from healthy individuals.

The first concentration was (0.2 ml) and the second was (0.1 ml), after harvesting the cultured cells and examination of the slides, it was found that the best concentration of PHA was (0.2 ml).

- Standardization of drugs doses:

In order to select the drug dose that cause inhibition of blastogenesis, mitosis or both, two concentrations of MTX (12.5 µg/ml, and 100µg/ml), and four concentrations of 6-TG (50 µg/ml, 100 µg/ml, 200 µg/ml, and 250 µg/ml) were prepared and tested for their effect on blood collected from (10) healthy individuals.

The experiments showed that the dose of (100 µg/ml MTX) can inhibit both BI and MI in all individuals.

For the 6-TG the dose of (250 µg/ml) inhibited both the BI, and MI in all individuals.

As a result, those two doses were used to test their effect on the lymphocytes of Bladder Cancer patients.

2.6.2: Assessment of Drug effects:

For each subject (Patient and control), 6 cultures were set up, three were used for the cytogenetic analysis (first group), and the other three (second group) were used for scoring MN, for each group, one tube was drug free, and the others were treated with the tested drugs.

2.7: Cytogenetic Analysis of Human Blood lymphocyte:

Blood cell culture for Cytogenetic analysis was performed following the procedure adapted by (Yunis, 1974)) with modification:

1. Human peripheral Blood was collected into heparin coated syringe.
2. Peripheral blood (0.25 ml) was added into test tube containing 2 ml of culture medium (RPMI- 1640).
3. PHA (0.25 ml) was added, the components were mixed gently.
4. The tubes were incubated at (37 °C) for (72 hours).
5. A portion of (0.2 mg/ml) of colchicine was added to each tube (2 hours) before harvesting.
6. The test tubes were centrifuged at speed of (2000 rpm) for (10 min).
7. The supernatant was removed and (5 ml) of KCl (hypotonic solution 0.075 M) was added, and then the test tubes were kept in the incubator at (37 °C) for (30 min) with shaking.
8. The tubes were centrifuged at (2000 rpm) for (10 min).

9. The supernatant was removed and the Carnoy's fixative was added as drops on the inside wall of the tubes with continuous shaking.
10. The cells were kept at (4 °C) for (30 min) for fixation.
11. The tubes were re- centrifuged at (2000 rpm) for (10 min.). The process was repeated 3 times and after that, the cells were suspended in (2 ml) of fixative solution.
12. A few drops from the tubes were dropped vertically by the use of Pasteur pipette from the height of 3 feet at a rate of (4 - 5) drops to give the chance for chromosomes to be spread well, then the slides were kept at room temperature.
13. The slides were stained with Giemsa stain for (30 min), and then washed with D.W.
14. Two slides for each concentration were prepared for Cytogenetic assay.

2.8: Micronucleus Test:

The method of Al- Sudany, 2005 was used for the MN test with modification:

1. Human blood was collected in a heparinized syringe.
2. (0.25 ml) of peripheral blood was added into test tube containing (2 ml) of culturing medium (RPMI- 1640).
3. (0.25 ml) of PHA was added. The components were mixed well.

4. (0.1 ml) of the prepared drug was added to each test tube.
5. The tubes were incubated at (37 °C), with gentle shaking every (24 hours); the incubation period was completed to 72 hours.
6. After passing (72 hour), the test tubes were centrifuged at speed of (800 rpm) for (5 min).
7. The supernatant was removed and (5 ml) of Potassium chloride (KCl) was added as a hypotonic solution at (0.1 M), then the test tubes were left for (30 min.) in the incubator at (37 °C) with continuous shaking.
8. The tubes were centrifuged at (800 rpm) for (5 min).
9. After that, the supernatant was removed and the Carnoy's fixative was added as drops on the inside wall of the test tube with continuous shaking, and then, the volume was fixed to (5 ml), and the contents were shaken well.
10. The tubes were kept at (4 °C) for (30 min.) to fix the cells.
11. The tubes were centrifuged once again at (800 rpm) for (5 min.), The process was repeated 3 times and after that, the supernatant was discarded and one drop of the pellet was smeared on a clean slide and left to dry at room temperature.
12. The slides were stained with Giemsa stain for (30 min.) then washed with D.W.
13. Two slides for each concentration were prepared for MN assay.

2.9: Cytogenetic Parameters Analysis:

2.9.1: Blastogenic index analysis (BI):

The slides were examined under light microscope and the Blastogenic index was determined as a ratio of blast cells (mitogen stimulated cells) to a total of 1000 growing cells. It was expressed as a percentage of the total cells using the following formula:

BI % = number of blast cells/ 1000 x 100

(AL- Shawk *et al*, 1999).

2.9.2: Mitotic index (MI):

The slides were examined under light microscope , the Mitotic index was determined by counting both the dividing and non

dividing cells as a ratio of mitotic cells to interphase nuclei in 1000 cells, and was expressed as a percentage of the total cells using the following formula:

MI % = number of dividing cells/ 1000 x 100

(Stich and San, 1981).

2.9.3: Chromosomal Aberrations (CA):

The prepared slides were examined under oil immersion lens for 100 dividing cells; the cells should be in the metaphase stage of the mitotic division where the chromosomal aberration are clear and the percentage of these aberration could be estimated.

2.9.4: Micronucleus (MN):

The number of MN in 2000 cells of lymphocytes was scored under the oil immersion lens, and the percentage of MN was calculated using the following formula:

Mn % = number of MN/ 2000 x 100

(Al- Sudany, 2005).

2.9.5: Statistical Analysis:

A one way analysis of variance was performed to test whether group variance was significant or not, the comparison between groups were used analysis of variance DENOVA (Al-Mohammed *et al*, 1986).

Chapter Three

Results and Discussion

Results and Discussion:

3.1: Drug Response:

In order to evaluate the response of the blood lymphocytes obtained from bladder cancer patients, the genotoxic effect of MTX and 6 -TG were tested. It is clearly showed that there is a significant

reduction in BI, and MI of cells treated with those drugs. The results are shown in table (3-1).

To picture the type of response of the cells to the drugs, a statistical method was adapted and this was previously described by (Al-Mohammed *et al*, 1986).

The patients were divided into three groups those groups were sensitive (S), moderately resistant (MR), and resistant (R).

The resistant group include patients whose lymphocytes showed continues blastogensis and mitosis when treated with the test drugs.

The S and MR groups were defined according to the concept of confidence interval (Upper and Lower Limits).

The lower was considered as a border line to separate the patients into S and R groups. All the patients who were below the border line were considered S and those who were above the border line were considered MR.

Table (3 -1)

Observed number of patients (S, MR, and R) treated with 6- TG and MTX

	6 - TG Response						MTX Response					
	Females		Males		Total		Females		Males		Total	
Patients Group	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%
S	0	0	6	40	6	33.3	2	67	2	13.4	4	22.3
MR	2	67	4	27	6	33.3	0	0	5	33.3	5	27.7
R	1	33	5	33	6	33.4	1	33.3	8	53.3	9	50
Total	3	100%	15	100%	18	100%	3	100%	15	100%	18	100%

S = sensitive, MR = moderately resistant, R = resistant.

Among 18 patients, (33.3 %) 6 patients were sensitive to 6- TG, the MR and R patients account for (33.3 %) and (33.4 %) respectively.

The male were more sensitive to 6- TG than females in which the sensitive males account for 40 % while the females account for 0 %. While the MR male account for 27 % and the female accounts for 67 % of the total MR patients.

The R male were account for 33.0 % just like the resistant females (33.3 %).

From those results it is clear that the male showed the highest sensitivity to the 6 - TG, the highest level of MR were showed by the females, while the resistant level were equal in both genders, the differences in the response of the gender to the drug may be of hormonal origin, due to the hormonal changes between the two

genders, or due to differences in the environmental exposure between the genders.

Among 18 BC patients, 4 Patients (22.3 %) were S to MTX, MR were *5* BC patients (27.7 %), and the R were 9 patients (50.0%).

The S females account for (67.0 %), while the S males account for 2 patients (13.4 %). While the MR female accounts for (0 %) while the MR males5 males account for (33.3 %), The R males were 8 patients account for (53.3 %), while the R females 1 patient account for (33.3 %)

The MTX is an anti - metabolite drugs, which act by inhibiting enzyme dihydrofolic acid reductase, that catalyses the conversion of folic acid to its active form folinic acid by binding to it (Duran *et al*, 2001). Resistance to MTX may occur due to certain mechanisms, these mechanisms include increase in DHFR enzyme level (due to DHFR gene amplification), or due to the impaired intracellular transport of MTX (Kunding *et al*, 1998; Serra *et al*, 2004).

The results showed that the females were highly sensitive to the MTX, while the males contribute the highest level of MR, and R to the drug, this may be occur due to the differences in the environmental exposure between the genders, or may of hormonal origin.

3.2: Cytogenetic Analysis of Drug Treated Lymphocytes:

3.2.1: MI of patients treated with 250 µg/ ml 6-TG:

The results of mitotic index (MI) (Mean± Standard error) of bladder cancer patients (S, MR, and R) and control in their response to 250 µg/ ml 6- TG are presented in the table (3- 3).

As shown for the MI value was dependent on the group of the patients (S, MR, and R).

When the lymphocytes of the BC patients and control cultured in culture media free from drug, it was shown that there were difference in the ratio of MI between the patients and the control, the control showed the highest level of MI, while the patients showed a lower rate of MI as shown in table (3-3).

But as an over all view there was no significant changes in the MI of the patients when compared with the MI of the control when they were cultured in media free from drugs.

When the cells were treated with 6-TG *in - vitro* the mitotic response of lymphocytes obtained from BC patients and control shows a similar manner in which the MI was reduced.

The MI of S group of patients increased from (0.28 ± 0.13) to (0.71± 0.03) about (60 %) induction, while for the control the MI reduced from (2.06 ± 0.34) to (0.88± 0.20), about (57 %), this induction in the MI was not significant when compared with the controls ($P > 0.05$).

The MR was reduced from (0.84± 0.09) to (0.50± 0.07) about (40 %) reduction, this reduction was not significant when compared with the control ($P > 0.05$).

The R group the MI was reduced from (2.58 ± 0.65) to (1.31± 0.55), about (49 %) reduction, this reduction was not very significant when compared with the control ($P > 0.05$).

Although the MI were increased slightly in both S, and MR groups of patients, but the induction was not significant.

The over all results showed that the MI was higher in patients than in the control when their lymphocytes treated with 6- TG.

The 6- TG resistant lymphocytes in normal human blood lymphocytes arise from somatic gene mutations (Albertini, 1985).

To understand the resistant mechanism we should first understand the mechanism of action of the 6- TG inside the living cell, the 6- TG are converted to thioguanine deoxy nucleotide triphosphate, it can substitute for deoxy guanosine tri phosphate (dGTP) in DNA polymerase reaction. This mechanism is thought to be the primary mechanism of cytotoxicity.

Resistances to 6 -TG usually occur due to mutations of the gene for hypoxanthine - guanine - ribosyl transferase (HGPRT) (Morley *et al*, 1983; Aubrecht *et al*, 1997), also the gene Hprt deficiency causes cellular resistance to 6 - TG (Dempsey *et al*, 1983)

Increased level of alkaline phosphatase which increases the break down of the nucleotide form is considered as one mechanism for 6-TG resistant (Beverly *et al*, 1981).

Usually patients receiving chemotherapy or radiation therapy showed significantly higher numbers of 6-TG resistant lymphocytes than healthy controls (Beverly *et al*, 1981).

3.2.2: MI of Lymphocytes treated with 100 µg/ml MTX:

The results of MI (Mean± standard error) of BC patients (S, MR, and R) and control when treated with MTX are shown in the table (3-4).

The MI values were dependent on the group of the patients (S, MR, and R).

As shown in the table the MI of S group of the BC patients treated with MTX were reduced from (0.28 ± 0.13) to (0.01± 0.002), about (96 %) reduction, while for the control the MI were reduced from (2.06 ± 0.34) to (0.36±0.007), about (82 %) reduction. This reduction was not significant ($P > 0.05$).

The mitotic index for the MR group of patients, was reduced from (0.84± 0.09) to (0.36±0.007), about (57 %) reduction.

The mitotic index for the R group, was reduced from (2.58 ± 0.65) to (0.68± 0.01), about (73 %) reduction, which was a highly significant change ($P< 0.05$).

The over all view showed that the MI in the patients was high when compared with the control when treated with the same drug.

The MTX is a potent inhibitor of dihydrofolate reductase (DHFR), which is a key enzyme for intracellular folate metabolism, and functions to regenerate tetrahydrofolate from dihydrofolate, a product

of thymidylate synthesis. as a consequence of DHFR inhibition, intracellular levels of tetrahydrofolate coenzymes are decreased, resulting in inhibition of thymidylate and consequently DNA biosynthesis, as well as purine synthesis (Bertino *et al*, 1996; Duran *et al*, 2001).).

Resistance to MTX may occur due to certain mechanisms. These mechanisms include increase in DHFR enzyme level (due to DHFR gene amplification), or due to the impaired intracellular transport of MTX , alteration (mutation) of DHFR that binds less avidly than the normal enzyme , and more recently the resistant may occur due to increased level of lysosomal enzyme (Bertino *et al*, 1996; Kunding *et al*, 1998; Serra *et al*, 2004).

As it is known, the DHFR participates in DNA, RNA, and protein synthesis; the mitosis is a part of the cell cycle which comes after the synthesis phase (S- phase), it means that the cells which reach to the M- phase expressed a certain process which may result from the resistance of the growing cells to the drug.

Those results agreed with the results of (Al- Amiry, 1999), there are many other chemotherapeutic drugs which cause reduction in the MI such like Tamoxifen (Al- Sudany, 2005), Metronidazole (MTZ) (Al- Romani, 2006).

3.2.3: BI of Lymphocytes treated with 250 µg/ml 6-TG:

The results of blastogenic index (BI) (Mean± S.E) of BC patients (S, MR, and R) and control in their response to 250 µg/ ml 6- TG *in vitro* are presented in the table (3- 3).

The 6- TG reduce the BI of lymphocytes obtained from BC patients and controls, the degree of reduction depends on the patients groups (S, MR, and R).

The 6- TG reduce the BI in the S group of patients from (12.05± 0.98) to (9.32± 0.70) about (22 %) reduction, while the reduction was higher in the control group from (26.94 ± 1.64) to (18.89± 1.4) about (30 %) reduction.

The MR also shows reduction in the BI from (19.57± 0.70) to (12.73± 0.78) about (35 %) reduction, while the R group also showed reduction in their BI from (25.03± 1.21) to (23.50± 2.94) about (6 %) reduction but their figure was higher than that of S, and the MR ($P < 0.05$).

As a general view, all the groups of the patients show blastogenic resistant to the 6- TG but as shown the reduction in the R group was much more higher than that of the S, and the MR groups, the highest reduction was among the R, then the MR showed the second highest level of reduction, the lowest reduction was among the S group of patients.

The lymphocyte blastogenic transformation occurs after stimulation with the mitogen (PHA), because the (PHA) is able to cross

link the lymphocyte receptors and induce the transformation (Morimoto and Wolff, 1980).

Reduction in the BI may occur due to mutations in the lymphocytes receptors involved in the mitogen recognition. The R patients may develop some mutations which render their cells resistant to the drug (Lange *et al*, 1981, Al- Abassi, 2001). Or the reduction in the BI may occur due to the cytotoxic effect of the 6-TG which is an analog of the natural purine (guanine), 6- TG is a substrate for the hypoxanthine- guanine phosphoribosyl transferase, and it is converted to the ribonucleotide 6- thioguanosine monophosphate, the accumulation of this monpphosphate inhibits several vital metabolic reactions (Elgemeie, 2003).

6- TG can both inhibit nucleotide and nucleic acid synthesis and be incorporated into nucleic acid (internet 8).

Those metabolic reactions may lead to the block of the purine biosynthesis pathway in side the living cell (Aubrecht *et al*, 1997).

This cytotoxic action of the 6- TG may cancel the effect of the PHA which led to the lymphocyte blastogenic transformation.

3.2.4: BI of Lymphocytes treated with 100 µg/ml MTX:

The results of BI (Mean± standard error) of BC patients (S, MR, and R) and control when treated with MTX are shown in the table (3-4).

As shown in the table the BI value were dependent on the group of the patients (S, MR, and R).

The BI for S BC patients treated with 100 µg/ml MTX was reduced from (12.9± 0.09) to (8.47± 0.30) but this reduction was highly significant (P <0.05) about (34 %) reduction, when compared with the control. While for the control group the BI reduced from (26.94 ± 1.64) to (20± 0.15) about (26 %) reduction.

The blastogenic index for the MR group the BI was reduced from (19.57± 0.7) to (14.88±0.10), about (24 %) reduction, this reduction was not highly significant when compared with the controls.

The blastogenic index for the R group of patients, the BI reduced from (25.03± 1.21) to (22.56± 0.28), about (10 %) reduction, this reduction was not significant when compared with the controls.

The MTX is used in cancer treatment due to its mode of action , MTX is a folate antagonist, it is act by inhibition of dihydrofolate reductase (DHFR), a key enzyme in the intracellular folate metabolism and it is essential for DNA synthesis and cell growth (Majumdar and Aggarwal, 2001;Serra *et al*, 2004).

Thus when the tumor cells treated with MTX, the malignant cells become starved for purine and pyrimidines, precursors for DNA and RNA required for proliferation (Majumdar and Aggarwal, 2001).

The resistant to MTX can arise through different mechanism, such as, decreased accumulation of the drug due to impaired transport, decreased retention of the drug, increase in the DHFR, altered DHFR that binds to MTX less avidly than the normal enzyme, and increased level of lysosomal enzyme (Rhee *et al*, 993; Bertino *et al*, 1996; Serra *et al*, 2004).

3.2.5: CA of lymphocytes obtained from BC patients and control:

The results of CA (Mean ± standard error) of BC patients (S, MR, and R) and control without any treatment are shown in the table (3 -2).

As shown for the parameters of BI, and MI, the CA value were dependent on the group of the patients (S, MR, and R).

The results showed that the total CA for the control were (0.09 ± 0.001), while for the patients, the total CA were (0.31 ± 0.06), about (34 %) induction, this induction was significant when compared with the control.

The chromosome break in the control were (0.04 ± 0.02), while for the S (0.00 ± 0.00), MR (0.00 ± 0.00), and the R (0.01 ± 0.01), about (75 %) reduction. Those reductions in the chromosomal breaks were not significant when compared with the control ($P < 0.05$).

The chromatid break for the control was (0.01 ± 0.008), while for the S (0.00 ± 0.00), MR (0.00 ± 0.00), and for the R (0.01 ± 0.01), those changes in the chromatid break was not significant when compared with the control ($P > 0.05$).

The gap for the control was (0.01 ± 0.008), while for the S (0.00 ± 0.00), for the MR (0.00 ± 0.00), and for the R (0.01 ± 0.01), those changes in the gap ratio was not significant when compared with the control ($P > 0.05$).

The deletion for the control was (0.01 ± 0.01), while for the S (0.15 ± 0.06), the MR (0.04 ± 0.01), and for the R (0.07 ± 0.03). The

highest level of the deletion was observed in the S group in which the ratio of the deletion increased for (15 fold) when compared with the control ($P < 0.05$), while for the MR and R the changes were not significant ($P > 0.05$).

The ring for the control was (0.00 ± 0.00), while for the S (0.00 ± 0.00), for the MR (0.01 ± 0.008), for the R (0.01 ± 0.01), those changes were not significant when compared with the control ($P< 0.05$).

The acentric for the control was (0.007 ± 0.005), while for the S (0.15 ± 0.06), about (15 fold) induction, in the MR (0.04 ± 0.01), about (47 %) induction, and for the R (0.08 ± 0.04), about (94 %) induction. The acentric showed significant level of changes in all group of patients when compared with the control ($P< 0.05$).

The dicentric for the control was (0.00 ± 0.00), while for the S (0.02 ± 0.02), for the MR (0.01 ± 0.008), and for the R (0.09 ± 0.06), about (9 folds) induction, the induction in the dicentric in the R group was highly significant when compared with the control ($P > 0.05$), while for the S, and MR the changes was not highly significant ($P < 0.05$).

The results showed that the CA in the patients were higher than CA in the control especially the MR group of patients, this is may due to the effect of the chemotherapy or radiation therapy the patients usually received.

3.2.6: CA for lymphocytes obtained from BC patients and control treated with 250 µg/ ml 6-TG in- Vitro:

The results of CA (Mean± S.E.) of BC patients (S, MR, and R) and control when treated with 6- TG are shown in the tables (3 - 3).

As shown for the parameters of BI, and MI, the CA values were dependent on the group of the patients (S, MR, and R).

In table (3- 4) the total CA for the BC patients and control are shown, while in the table (3- 7), the CA are shown in details.

It was found that the total CA for S patients treated with 6- TG was reduced from (0.071± 0.03) to (0.02±0.02), about (7 %) reduction, while for the control the CA were increased after the treatment from (0.09 ± 0.001) to (0.12± 0.05), about (25 %) induction. This reduction in the S patients was not significant when compared with the control ($P > 0.05$).

For the MR group the CA was reduced slightly from (0.28± 2.22) to (0.20± 0.02), about (28 %) reduction, in which this reduction was not significant when compared with the control ($P > 0.05$).

While for the R group the CA was increased from (0.63± 0.08) to (0.72± 0.08), about (14 %) induction. This induction was highly significant when compared with the control ($P < 0.05$).

The detailed CA for the BC patients and control are shown in table (3-7). The chromosome breaks for the control were (0.08 ± 0.04) which was higher than the ratio of chromosome break in the untreated control (0.04 ± 0.02), the rate of induction was not significant. For the S group the chromosome break were (0.00 ± 0.00), for the MR group was induced from (0.00 ±0.00) to (0.52 ± 0.03), this induction was not significant when compared with the control, for the R group the chromosome break induced from (0.01 ± 0.01) to (0.2 ± 0.007). This

induction was not significant when compared with the control (P< 0.05).

The chromatid breaks for the control were reduced from (0.007 ± 0.005) to (0.01 ± 0.008), about (30 %) reduction, while for the S the ratio was the same (0.00 ± 0.00), and the MR the chromatid breaks induced from (0.00 ± 0.00) to (0.05 ± 0.03), and for the R the chromatid breaks induced from (0.01 ± 0.01) to (0.02 ± 0.007), those changes in the chromatid break in the S and R groups of patients were not significant when compared with the control (P > 0.05), while for the MR group the chromatid break were significant when compared with the control (P < 0.05).

The gap for the control was reduced from (0.01 ± 0.008) to (0.007 ± 0.005), about (30 %) reduction, while for the S group of patients, the ratio was the same (0.00 ± 0.00), for the MR the gap induced from (0.00 ± 0.00) to (0.05 ± 0.03), and for the R was induced from (0.01 ± 0.01) to (0.02 ± 0.007), those changes in the gap ratio for the Sand R group of patients were not significant when compared with the control (P >0.05), while for the MR group the changes were significant (P < 0.05).

The deletion for the control was reduced from (0.01 ± 0.01) to (0.007 ± 0.007), about (30 %) reduction, while for the S reduced from (0.15 ± 0.06) to (0.11 ± 0.06), about (30 %) reduction, which was not significant when compared with the control. In the MR group induced from (0.04 ± 0.01) to (0.07 ± 0.05), about (40 %) induction, and for the R induced from (0.07 ± 0.03) to (0.11 ± 0.04), about (40 %) induction.

The MR and R group showed a significant rate of changes when compared with the control ($P < 0.05$).

The ring for the control was the same (0.00 ± 0.00), for the S group the ratio was the same (0.00 ± 0.00), for the MR the ring induced from (0.01 ± 0.008) to (0.02 ± 0.01), this induction was significant when compared with the control, for the R the ring reduced from (0.01 ± 0.01) to (0.008 ± 0.007), this change was not significant when compared with the control ($P > 0.05$).

The acentric for the control was induced from (0.007 ± 0.005) to (0.02 ± 0.013), about (65 %) induction, while for the S it was reduced from (0.15 ± 0.06) to (0.11 ± 0.06), about (25 %) reduction, for the MR it was induced from (0.04 ± 0.01) to (0.07 ± 0.04), about (40 %) induction, and for the R it was induced from (0.08 ± 0.04) to (0.13 ± 0.05), about (40 %) induction. The acentric showed significant level of changes in the MR and R group of patients when compared with the control ($P < 0.05$), while for the S group, the changes was not significant when compared with the control ($P > 0.05$).

The dicentric for the control was induced from (0.00 ± 0.00) to (0.02 ± 0.01), which was highly significant, while for the S it was reduced from (0.02 ± 0.02) to (0.00 ± 0.00), for the MR the dicentric ratio was the same (0.01 ± 0.008), and for the R it was reduced from (0.09 ± 0.06) to (0.03 ± 0.03), the changes in the dicentric in the patients group was not significant when compared with the control ($P > 0.05$).

The 6- TG resistant lymphocytes in normal human blood lymphocytes arise from somatic gene mutations (Albertini, 1985).

To understand the resistance mechanism we should first understand the mechanism of action of the 6- TG inside the living cell, the 6- TG are converted to thioguanine deoxy nucleotide triphosphate, it can substitute deoxy guanosine tri phosphate (dGTP) in DNA polymerase reaction. This mechanism is thought to be the primary mechanism of cytotoxicity.

Resistances to 6TG usually occur due to mutations of the gene for hypoxanthine - guanine - ribosyl transferase (HGPRT) (Morley *et al*, 1983; Aubrecht *et al*, 1997).

Increased level of alkaline phosphatase which increase the break down of the nucleotide form consider as one mechanism for 6-TG resistance (Beverly *et al*, 1981).

Usually patients receiving chemotherapy or radiation therapy showed significantly higher numbers of 6-TG resistant lymphocytes than healthy controls (Beverly *et al*, 1981).

the results clarify that the MR, and R groups showed high levels of CA, this result is normal due to the toxicity of the drug, in which the MR, and R groups had pass the S- phase and reached to the M- phase, under the toxic effect of 6 –TG which act usually to stop the cell proliferation, this means that the cells which reach the M- Phase had pass the toxic effect of the drug which cause certain damages to the DNA, which in turn show high level of CA.

3.2.7: CA of Lymphocytes obtained from BC patients and control treated with 100 µg/ml MTX:

The results of CA (Mean± standard error) of BC patients (S, MR, and R) and control in their response to MTX are shown in the tables (3-4) and table.

As shown in the tables the CA value was dependent on the group of the patients (S, MR, and R).

As shown in the table, the CA of S group of BC patients was induced from (0.071± 0.03) to (0.30± 0.03), about (60 %) induction, but this induction was not significant when compared with the control ($P > 0.05$).

The CA for the control group reduced from (0.90 ± 0.001) to (0.19± 0.37), about (80 %) reduction.

For the MR group of patients the MI was induced from (0.28± 2.22) to (0.43± 0.01), about (35 %) induction, which is not a significant increase when compared with the control.

For the R group of patients, the CA was induced from (0.63± 0.08) to (1.28± 0.03), about (50 %) induction.

The detailed CA for the BC patients and control when treated with MTX are shown in table (3-8), the chromosome break for the control were induced from (0.04 ± 0.02) to (0.11 ± 0.03), about (60 %) induction, for the S the Chromosome breaks were induced from (0.00 ± 0.00) to (0.16 ± 0.06), which was not significant when compared with the control, for the MR was induced from (0.00 ±0.00) to (0.10 ± 0.04), this induction was not significant when compared

with the control, and for the R the chromosome breaks were induced from (0.01 ± 0.01) to (0.14 ± 0.13), this induction was not significant when compared with the control (P > 0.05).

The chromatid break for the control were induced from (0.007 ± 0.005) to (0.03 ± 0.01), about (75 %) induction, while for the S the ratio was induced from (0.00 ± 0.00) to (0.23 ± 0.15),this induction was very significant when compared with the control, and the MR group the chromatid break induced from (0.00 ± 0.00) to (0.11 ± 0.05), which was significant when compared with the control, and for the R the chromatid break induced from (0.01 ± 0.01) to (0.16 ± 0.16), about (90 %) induction, which was highly significant when compared with the control (P > 0.05).

The gap for the control was induced from (0.01 ± 0.008) to (0.03 ± 0.01), about (65 %) induction, while for the S group of patients, the ratio was induced from (0.00 ± 0.00) to (0.23 ± 0.15),which was highly significant when compared with the control (P < 0.05), for the MR the gap induced from (0.00 ± 0.00) to (0.11 ± 0.05), and for the R was induced from (0.01 ± 0.01) to (0.16 ± 0.16), about (90 %) induction, the changes in the gap ratio in all groups of patients were highly significant when compared with the control.

The deletion for the control was reduced from (0.01 ± 0.01) to (0.007 ± 0.007), about (30 %) reduction, while for the S reduced from (0.15 ± 0.06) to (0.11 ± 0.06), about (30 %) reduction, which was not significant when compared with the control, in the MR group induced from (0.04 ± 0.01) to (0.07 ± 0.05), about (40 %) induction,

and for the R induced from (0.07 ± 0.03) to (0.11 ± 0.04), about (40 %) induction, the MR and R group showed a significant rate of changes when compared with the control ($P < 0.05$).

The ring for the control was induced from (0.00 ± 0.00) to (0.007 ± 0.14), for the S group the ratio was the same (0.00 ± 0.00), for the MR the ring induced from (0.01 ± 0.008) to (0.04 ± 0.03), about (75 %) induction, this induction was significant when compared with the control ($P< 0.05$), for the R group the rings were reduced from (0.01 ± 0.01) to (0.00 ± 0.00), this change was not significant when compared with the control ($P> 0.05$).

The acentric for the control was induced from (0.007 ± 0.005) to (0.02 ± 0.01), about (65 %) induction, while for the S group it was reduced from (0.15 ± 0.06) to (0.00 ± 0.00), for the MR group it was induced from (0.04 ± 0.01) to (0.01 ± 0.008), about (75 %) induction, and for the R it was induced from (0.08 ± 0.04) to (0.13 ± 0.05), about (40 %) induction. The acentric showed significant level of changes in the MR and R group of patients when compared with the control ($P< 0.05$), while for the S group, the changes was not significant when compared with the control ($P > 0.05$).

The dicentric for the control was induced from (0.00 ± 0.00) to (0.02 ± 0.01), which was highly significant, while for the S it was reduced from (0.02 ± 0.02) to (0.00 ± 0.00), for the MR group the dicentric ratio was the same (0.01 ± 0.008), and for the R it was reduced from (0.09 ± 0.06) to (0.04 ± 0.04), about (55 %), the

changes in the dicentric in the patients group was not significant when compared with the control ($P > 0.05$).

The high level of CA is a clear sign of the high toxicity of the MTX to the living cells, how MTX mediates its effects at the cellular level is not fully under- stood. MTX is taken up by cells and tissues and converted to MTX- polyglutamtes, long – lived derivatives that retain biochemical and biological activity within the cell. MTX – polyglutamates can competitively inhibit dihydrofolate reductase (DHFR), which ultimately affects both purine and pyrimidine nucleotide biosynthesis. Thus during the treatment of cancer with MTX, the malignant cells become starved for purine and pyrimidines, precursors of DNA and RNA required for proliferation (Shaker *et al*, 2001; Serra *et al*, 2004).

Resistance to MTX may occur due to certain mechanisms, these mechanisms include increase in DHFR enzyme level (due to DHFR gene amplification), or due to the impaired intracellular transport of MTX (Kunding *et al*, 1998; Serra *et al*, 2004).

Table (3 - 2)

Cytogenetic analysis and chromosomal aberrations of lymphocytes obtained from bladder cancer patients and control, differences A, B, C are Significant (P≤ 0.05)

	Lymphocytes obtained from BC patients and control									
	BI	MI	CA	Chromosome break	Chromatid Break	Gap	Deletion	Ring	Acentric	Dicentric
Control	26.94 ± 1.64 A	2.06 ± 0.34 A	0.09± 0.001 A	0.04 ± 0.02 A	0.01 ±0.008 A	0.01±0.008 A	0.01± 0.01 A	0.00±0.00 A	0.007 ±0.005 A	0.00 ± 0.00 A
S	12.05± .0.98 B	0.28 ± 0.13 B	0.071± 0.03 A	0.00 ± 0.00 A	0.00 ± 0.00 A	0.00± 0.00 A	0.15± 0.06 B	0.00±0.00 A	0.15 ± 0.06 C	0.02 ± 0.02 A
MR	19.57± 0.70 C	0.84± 0.09 AB	0.28 ± 0.35 B	0.00 ± 0.00 A	0.00 ± 0.00 A	0.00± 0.00 A	0.04 ± 0.01 A	0.01±0.008 A	0.04 ± 0.01 AB	0.01 ± 0.008 A
R	25.03± 1.21 AC	2.58 ± 0.65 A	0.63 ± 0.08 C	0.01 ± 0.01 A	0.01 ± 0.01 A	0.01±0.01 A	0.07± 0.03 A	0.01 ± 0.01 A	0.08 ± 0.04 B	0.09 ± 0.06 B

S = sensitive, MR= moderately resistant, R= resistant, BC = Bladder cancer, BI= blastogenic index, MI= mitotic index, CA= chromosomal aberrations

Table (3 - 3)

Cytogenetic analysis and chromosomal aberrations of lymphocytes obtained from BC patients and control treated with 250 µg/ml 6- TG in – Vitro

	Lymphocytes obtained from BC patients and control treated with 250 µg/ml 6- TG in- Vitro									
	MI	BI	CA	Chromosome break	Chromatid Break	Gap	Deletion	Ring	Acentric	Dicentric
Control	0.88±0.20 A	18.89±1.4 A	0.12±0.05 A	0.08 ± 0.04 A	0.007± 0.005 A	0.007± 0.005 A	0.007 ± 0.007 A	0.00 ± 0.00 A	0.02 ± 0.013 B	0.02 ± 0.01 B
S	0.71± 0.03 A	9.32± 0.70 B	0.02± 0.02 A	0.00 ± 0.00 A	0.00 ± 0.00 A	0.00 ± 0.00 A	0.11 ± 0.06 A	0.00 ± 0.00 A	0.11 ± 0.06 A	0.00 ± 0.00 A
MR	0.50± 0.07 A	12.73±0.78 B	0.20 ± 0.02 A	0.52 ± 0.03 A	0.05 ± 0.03 B	0.05 ± 0.03 B	0.07 ± 0.05 B	0.02 ± 0.01 B	0.07 ± 0.04 B	0.01± 0.008 A
R	1.31± 0.55 A	23.50± 2.94 C	0.72± 0.08 B	0.2 ± 0.007 A	0.02 ± 0.007 A	0.02 ± 0.007 A	0.11 ± 0.04 B	0.008 ± 0.007 A	0.13 ± 0.05 B	0.03± 0.03 A

S = sensitive, MR= moderately resistant, R= resistant, BC = Bladder cancer, BI= blastogenic index, MI= mitotic index, CA= chromosomal aberrations

BI= blastogenic index, MI= mitotic index, CA= chromosomal aberrations

Table (3 - 4)

Cytogenetic analysis and chromosomal aberrations of lymphocytes obtained from BC patients and control treated with 100 µg/ ml MTX in- Vitro

	Lymphocytes obtained from BC patients and control									
	MI	BI	CA	Chromosome break	Chromatid Break	Gap	Deletion	Ring	Acentric	Dicentric
Control	0.36±0.007 A	20.0±0.15 A	0.19±0.37 A	0.11 ± 0.03 A	0.03 ± 0.01A	0.03 ± 0.01 A	0.01 ± 0.008 A	0.007 ± 0.14 A	0.02 ± 0.01 A	0.01 ± 0.008 A
S	0.01± 0.002 B	8.47± 0.30 B	0.30 ± 0.03 A	0.16 ± 0.06 A	0.23 ± 0.15 B	0.23 ± 0.15 B	0.00 ± 0.00 A	0.00 ± 0.00 A	0.00 ± 0.00 A	0.02 ± 0.01 A
MR	0.28 ± 0.01 AB	19.57± 0.70 C	0.43± 0.03 A	0.10 ± 0.04A	0.11 ± 0.05 AB	0.11 ± 0.05 AB	0.01 ± 0.009 A	0.04 ± 0.03 B	0.01 ± 0.008 A	0.39 ± 0.36 B
R	0.68± 0.01 C	22.56± .023 A	1.26±0.03 B	0.14 ± 0.13 A	0.16 ± 0.16 AB	0.16 ± 0.16 AB	0.00 ± 0.00	0.00 ± 0.00 A	0.04 ± 0.04 A	0.00± 0.00 A

S = Sensitive, MR= Moderately Resistant, R= Resistant, BC = Bladder Cancer.

BI= blastogenic index, MI= mitotic index, CA= chromosomal aberrations

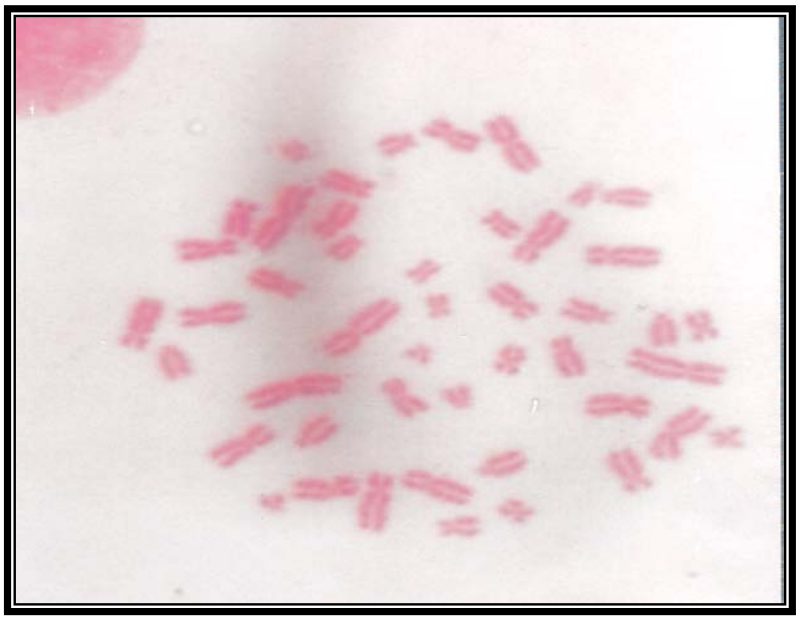


Figure (3 -1)

Metaphase of normal human blood lymphocytes

(1000X)

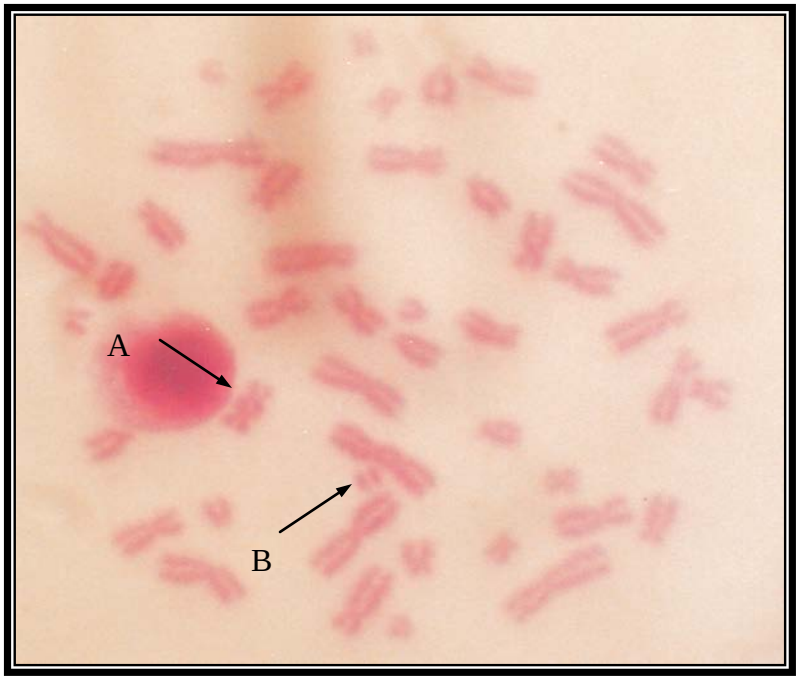


Figure (3-2)

Metaphase of human lymphocytes obtained from bladder cancer patients treated with 250µg/ ml 6-TG showing:

A -dicentrinc, and (B) a centric chromosomes

(1000X)

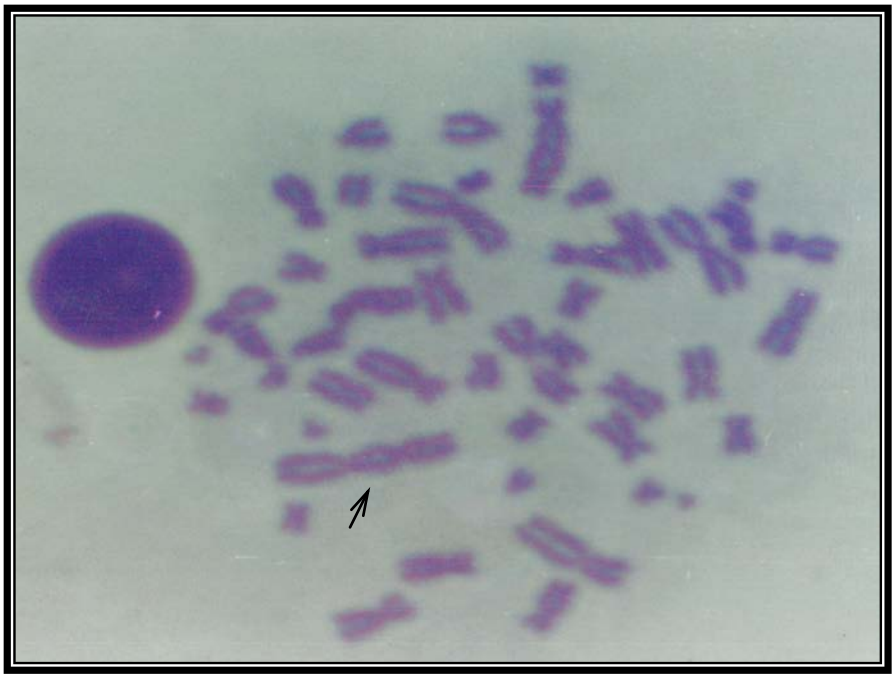


Figure (3-3)

Metaphase of human lymphocytes obtained from bladder cancer patients treated with 250µg/ ml 6-TG showing:

dicentrinc.

(1000X)

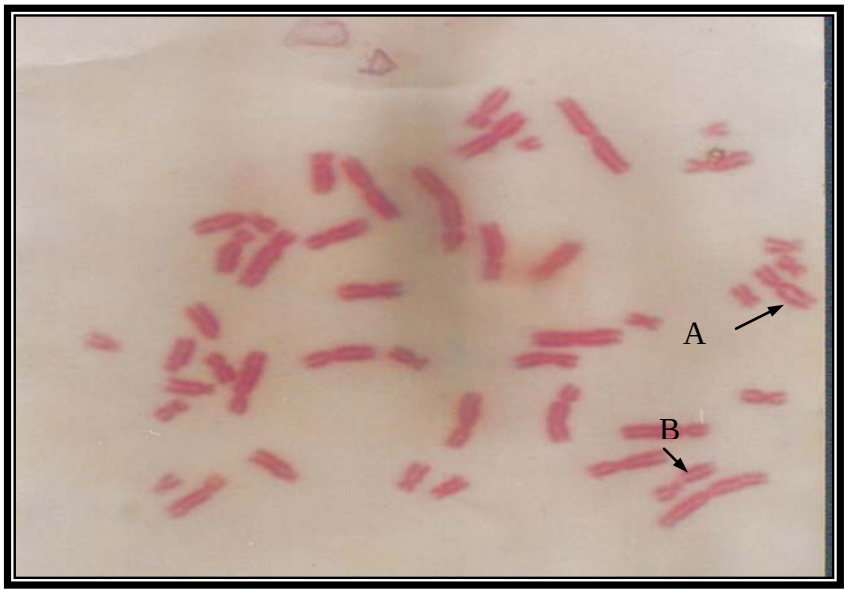


Figure (3-4)

Metaphase of human lymphocyte obtained from bladder cancer patients treated with 100 µg/ml MTX showing: (A) gap and, (B) a centric chromosomes

(1000X)

3.2.8: MN assay for BC patients treated with drugs:

3.2.8. A: MN assay for BC patients and control:

The results of MN (Mean± standard error) of BC patients (S, MR, and R) and control are shown in table (3- 5).

As shown in the table the MN value were dependent on the group of the patients (S, MR, and R).

The MN test is an easy, rapid, and sensitive test used to determine the damages of the DNA when treated with different agents (Koteles *et al*, 1993). Micronuclei are cytoplasmic chromatin

masses with the appearance of small nuclei that arise from chromosomes lagging at anaphase or from acentric chromosomal fragments (Al- Sudany, 2005).

It was found that all chromatide, chromosome, and isochromaid breaks, as well as a symmetrical and incomplete symmetrical exchange, will give rise to acentric fragments at mitosis, and that these fragments are frequently excluded from, the daughter nuclei and appear in the following interphase as micronucleus (Heddle *et al*, 1983).

There are two procedures for the MN assay, the first one which include the addition of Cytochalasin B in to the chromosome medium, after the stimulation of the cultured cells with the PHA, the harvesting are done by the same manner which include treatment with hypotonic then fixation of the cells, the slides are stained with acridine orange (Rothfuss *et al*, 2000).

The method for measuring the MN in this study include the use of the same culturing method with use of the media RPMI-1640, the difference was in the concentration of the hypotonic solution which was (0.01 M KCL).

As shown in the table (3- 5) and (3- 6), it is clear that there is no significant changes in the ratio of MN between the patients and control, the MN for control was (0.24± 0.10), while for the patients was (0.31± 0.005), without any treatment.

For the S group of patients the MN was (0.20± 0.03), and for the MR group (0.49± 0.07), and for the R group the MN (0.39± 0.10), the ratio of induction for all the groups of patients were not very significant when compared with the control (P>0.05).

3.2.8. B: MN assay for BC patients treated with 250 µg/ml 6-TG:

The results of MN (Mean± standard error) of BC patients (S, MR, and R) and control in their response to 250 µg/ml 6- TG are shown in the table (3-6).

As shown in the table the MN value were dependent on the group of the patients (S, MR, and R).

The result of the MN in lymphocytes from the S patients with BC (Mean ± S. E) when treated with 250 µg/ml 6- TG were changed from (0.20± 0.03) to (0.20± 0.01), which was not significant (P> 0.05).

For the control MN were induced from (0.24± 0.10) to (0.27± 0.009), about (11 %), this induction was not significant (P> 0.05).

For the MR group the MN were reduced slightly from (0.49± 0.07) to (0.32± 0.003), about (35 %) reduction, this reduction was not significant when compared with the control (P > 0.05).

For the R group of patients, the MN was induced from (0.39± 0.10) to (0.62 ± 0.01), about (37 %) but this induction when compared with the control was not significant (P> 0.05).

Those results may lead to the conclusion that the 6- TG has no effect on the MN induction in BC patients.

3.2.8. C: MN assay for BC patients treated with 100 µg/ml MTX:

The results of MN (Mean± standard error) of BC patients (S, MR, and R) and control in their response to MTX are shown in the table (3-6).

Which indicate that the MN value were dependent on the group of the patients (S, MR, and R).

The results of the MN for the S group of patients (Mean ± S. E) of BC patients when treated with MTX were induced from (0.20± 0.03) to (0.25± 0.008), about (20 %) induction. This induction was not significant when compared with the control (P> 0.05).

The MN for the control was induced by MTX treatment from (0.24± 0.10) to (0.32 ± 0.01) about (25 %) induction, which was not significant (P< 0.05).

For the MR group of patients the MN reduced from (0.49± 0.07) to (0.36± 0.008), about (26 %) reduction, which was significant when compared with the control.

For the R group of patients the MN was induced when the lymphocytes were treated with MTX from (0.39± 0.10) to (0.72±

0.04), about (45%) induction, this induction was highly significant when compared with the control.

These results showed that the MTX is highly toxic to the living cells, and it induces the formation of MN in the lymphocytes from the BC patients.

As shown in the result the MTX increase the level of MN in both MR, and R group of patients, this means that the MTX trigger the fragmentation of the chromosomes which in turn lead to the formation of MN in the MR, and R group which pass the S- phase of the cell cycle due to their resistance.

Table (3-5)

The MN Analysis of BC patients and controls

(Mean± S.E.)

Differences A, B, C are Significant (P< 0.05)

MN	Control	BC Patients
untreated	0.24± 0.10 A	0.31± 0.005 A
6- TG	0.26± 0.003 A	0.38± 0.006 A
MTX	0.32± 0.01 A	0.36± 0.005 A

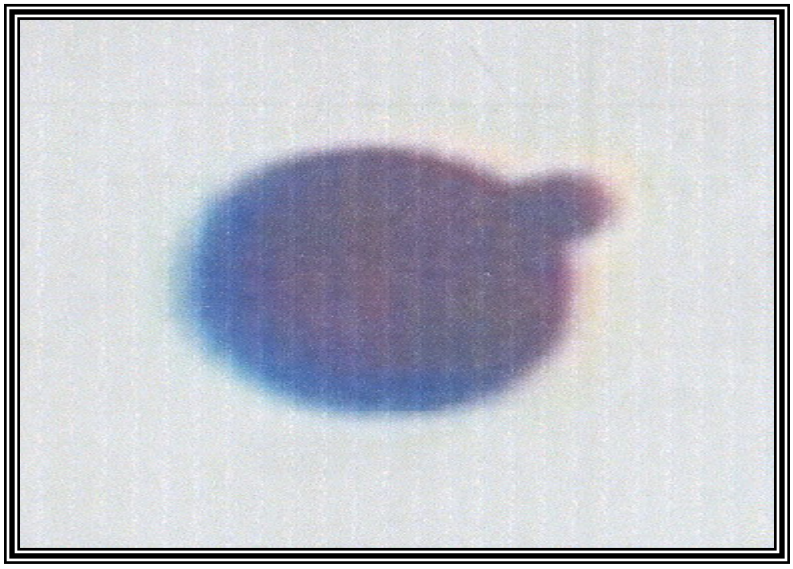
Table (3– 6)

The MN of BC patients and controls treated with drugs (Mean ± S.E)

Differences A, B, C are Significant (P< 0.05)

MN	Control	BC Patients			
		Mean± S.E	S	MR	R
Untreated	0.24± 0.10 A	0.31±0.005 A	0.20± 0.03 A	0.49± 0.07 A	0.39± 0.10 A
6- TG	0.27±0.009 A	0.38±0.006 A	0.20± 0.01 A	0.32± 0.003 A	0.62± 0.01 A

MTX	0.32 ± 0.01 A	0.36±0.005 A	0.25±0.008 A	0.36± 0.008 AB	0.72± 0.04 B



(3-5)

Human lymphocytes obtained from bladder cancer patients treated with 100 µg/ml MTX showing Micronucleus

(1000 X)

Table (3-7)

Total changes of the studied parameters

Total Changes				
		BC Patients		
Parameter	Control	S	MR	R
BI Control	26.9±1.64	12.05±0.98	19.57±0.70	25.03±1.21
BI with 6- TG	18.89±1.4	9.32±0.70	12.73±0.78	23.5±2.94
BI with MTX	20.0±0.15	8.47±0.30	14.88±0.10	22.56±0.23
MI Control	2.06±0.34	0.28±0.13	0.84±0.09	2.58±0.65
MI with 6- TG	0.88±0.20	0.71±0.03	0.50±0.07	1.31±0.55
MI with MTX	0.36±0.00	0.01±0.002	0.28±0.01	0.68±0.01
CA Control	0.09±0.001	0.07±0.03	0.28±0.35	0.63±0.08
CA with 6- TG	0.12±0.34	0.02±0.02	0.20±0.02	0.72±0.08
CA with MTX	0.19±0.37	0.30±0.03	0.43±0.03	1.26±0.03
MN Control	0.24±0.10	0.20±0.03	0.49±0.07	0.39±0.10

MN with 6- TG	0.27±0.009	0.20±0.01	0.32±0.003	0.62±0.01
MN with MTX	0.32±0.01	0.25±0.00	0.36±0.008	0.72±0.04

Chapter Four

Conclusions and Recommendations

4.1: Conclusion:

- The males with BC showed the highest level of resistance to both drugs 6- TG and MTX, while the females with BC showed moderate resistance to both drugs 6- TG and MTX.
- 6- TG and MTX reduce the levels of MI, and BI in all groups of patients (S, MR, and R).
- The 6- TG treated cells showed slight increase in the CA, while the MTX has no effect on the CA of the BC patients.
- There is no relation between the MN incidence in the BC patients and the treatment with the 6- TG, while The MTX treated lymphocytes showed increased level of MN in R group of patients.

4.2: Recommendations:

- Study the genotoxic effect of the 6- TG, and MTX on biopsies obtained from the bladder of the BC patients.
- Study the SCE on the lymphocytes as well as the biopsies of the bladder in the BC patients when treated with 6- TG as well as MTX.
- Study the effect of other drugs which are used in the treatment of cancers on the lymphocytes as well as the biopsies of the patients.

Chapter Five

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الخلاصة :

تضمن الدراسة الحاليه الكشف عن التاثيرات الوراثية الخلوية لمادتي ٦- ثايوكوانين و ميثوتركسيت في الخلايا اللمفاوية لمرضى سرطان المثانه في الانسان، حيث درست نماذج الدم المأخوذة من عينه عشوائية و قورنت النتائج المرضية مع عينه قياسية (غير مريضة) مماثله لها.

أستعملت في هذه الدراسة اربع مؤشرات وراثية و هي مؤشر انقسام الخيطي و معامل الاورومي و التشوهات الكروموسومية و معامل الانوية الصغيرة .

و اظهرت النتائج ما يلي:

١. أعلى نسبة مقاومة للعقارين المستدخدمين سجلت في الذكور.
٢. عندما عوملت خلايا المرضى بالعقارين المستخدمين (ميثوتركسيت و ٦- ثايوكوانين) تم اختزال معامل الانقسام الخيطي ، و عند مقارنة هذه النتائج مع السيطرة السالبة ، اظهر المرضى مقومة لكل من المثوتركسيت و ٦- ثاوكوانين.
٣. عندما عوملت خلايا المرضى بالعقارين المستخدمين (ميثوتركسيت و ٦- ثايوكوانين) تم اختزال معامل الاورومية و عند مقارنة هذه النتائج مع السيطرة السالبة ، اظهر المرضى مقومة لكل من المثوتركسيت و ٦- ثاوكوانين.
٤. تكرارات التغييرات الكروموسوميه لخلايا المرضى ازدادت قليلا عندما عوملت بعقار ٦- ثيوكوانين ، و عند معامله خلايا المرضى بعقار الميثوتركيست لم تلاحظ اي تغيرات في الكروموسومات.
٥. تكرارات الانوية الصغيرة في خلايا المفاوية للمرضى قد فحصت بعد المعالجة بعقاري ٦- ثايوكوانين، و المثوتركسيت، لا توجد هناك اختلافات جوهرية في الانوية الصغيرة قبل و بعد المعالجه بالعقارين.

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و هي جزء من متطلبات نيل درجه الماجستير علوم في التقانه الاحيائية

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Al- Nahrain University/ College of Science
Biotechnology Department

Cytogenetic effects of 6 – TG and MTX on human blood lymphocytes from bladder cancer patients

A thesis

Submitted to the college of Science of Al- Nahrain University in a partial of fulfillment of the requirements for the degree of Master of Science in Biotechnology

By

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B.Sc. Biotechnology/ Al- Nahrain University 2002

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الى.....

ينبوعا الحنان و رمزا العطاء الدائم

والدي و و الدتي ..... تقديرا و اعتزازا

من اكن لهم كل الحب و الاحترام

اختي و اخي .....

من دفعني دوما كي اكون باحثة

خطيبي بهاء.....

الى كل من اوقد شمعه انارت طريق حياتي....

اهدي بحثي هذا

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Summary:

This study included the reveal of the Cytogenetic effects of 6-Thioguanine and methotrexate in lymphocytes obtained from bladder cancer patients, in which we study the effects of those drugs on the blood samples obtained from patients and apparently health individuals, the patients were diagnosed in Al- Yarmouk Hospital.

In this study Mitotic index, blastogenic index, chromosomal aberrations, and micronucleus, were employed in the present study as cytogenetic parameters for the mentioned drugs. The most prominent findings of the present work were:

1. The males with bladder cancer showed the highest level of resistant to both drugs.
2. When the patients' cells were treated with 6 - TG, and MTX *in – vitro*, it reduced MI, but as an over all view, the patients show resistance to 6- TG, and MTX when compared with the control group.
3. When the patients' cells were treated with 6 –TG and MTX *in – vitro*, it reduced the BI, but as an over all view, the patients show resistance to 6- TG, and MTX when compared with the control group.

4. The chromosomal aberrations frequencies of the patients had increased slightly when treated with the 6- TG.
5. When the patients were treated with MTX, no significant increase in the chromosomal aberrations was observed.
6. The Micronuclei frequency was scored for the patients after treatment with the 6- TG and MTX *in – vitro* where there was no significant change in its ratio.

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List of Abbreviation

List of Abbreviations	
6- TG	6- Thioguanine
BI	Balstogenic index
CA	Chromosomal aberrations
DHFR	Dihydrofolate reductase
DNA	Deoxy ribonuclic acid
Hprt	Hypoxanthine phosphoribosyl transferase
MI	Mitotic index
MN	Micronucleus

MR	Moderately resistant
MTX	Methotrexate
PHA	Phytoheamagglutinine
R	Resistant
S	Sensitive