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Cytogenetic studies of the drug methotrexate (MTX) on the blood lymphocytes of colon cancer patients .

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Abstract: The aim of study is to investigate the cytogenetic parameters in order to define the effect of methotrexate (MTX) on blood lymphocytes, manifested by blast index (BI), mitotic index (MI), replicative index (RI), and sister chromatid exchanges (SCEs). The results showed significant reduction in BI and MI and RI indices with MTX concentrations (0.0, 0.2 ,0.5 ,1, 2, 4, 8, µg/ml) in comparison with the control. The results of BI showed significant reduction according to increasing of concentration. Of the drug in colon cancer patients, it gave 28.6 %, 18.6 %, 14.5%, 10.4%, 6.8 % , 6.7 %, 3.3% respectively. The results of (MI) gave 3.6 %, 2.3%, 1.6%, 0.18%, 0.22%, 0.0%, 0.0 % respectively. The values of (RI), gave 0.72% at zero concentration and 0.44% at concentration of 0.2µg/ml of M% TX and 0.27 % at concentration of 0.5µg/ml. On the other hand the SCE showed increases in the SCEs frequency in colon cancer samples in MTX concentration (0.0, 0.2, 0.5, µg/ml) which gave 8.47, 10.41, 10.49. respectively. The SCEs in the high concentration of drug (1,2,4,8 µg/ml) cannot be detected due to its toxicity of the drug. The mutation fraction increased in colon cancer patient which gave 9 at 0.2 µg/ml, and 15.72 at 0.5 µg/ml and 4.1 in 1 µg/ml. The conclusion demonstrated decreasing in (MI), (BI) and (RI) and increasing in the SCEs frequencies and mutation fraction in colon cancer patients in comparison with the control. The presence of mutant cells in lymphocytes of colon cancer that resistant to MTX, gave an idea about amplification in DHFR gene that regulate the activity of DHFR enzyme and also indicated the resistance of cancer cells to drugs.

Key words: Methotrexate. Colon cancer , Cytogenetic analysis.

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Introduction:

Worldwide, more than one million individuals develop colorectal cancer (CRC) each year, and the disease-specific mortality rate is nearly 33% in the developed world(1). Colorectal cancer most commonly occurs sporadically, and only 25% of the patients have a family history of the disease, suggesting a contribution for hared genes and environment. However, only 5%-6% of CRC is due to inherited mutations in major CRC genes whilst the remaining of the familial forms likely result from gene environment interactions (2). The development of colorectal cancer is a multistep process that involves an accumulation of mutations in tumor suppressor genes and oncogenes. It has provided a useful model for the understanding of the multistep process of carcinogenesis. The model of colorectal tumorigenesis

includes several genetic changes that are required for cancer initiation and progression (3).The development of CRC is induced by a combination of genetic and environmental factors , whose study is essential for the establishment of new prevention strategies(4).

For colorectal cancers, the acquisition of genomic instability is considered a key hallmark . Three major molecular subtypes can be recognized : MN ((or MSI, for microsatellite instability).CIN (for chromosomal instability) and CIMP (for CpG island methylator phenotype).CIN is the most common type of genomic instability observed in colon cancer and occurs in 80-85% of colorectal cancers(5). CIN in CRC characterized mainly by chromosomal rearrangements and numerical abnormalities at a greatly increased rate compared with normal cells (6). Medical reports have shown that chromosomal abnormalities and microsatellite are early in the development of the tumor which support the hypothesis of phenotype hypothesis , which shows that there are multiple pathways in maintaining gene stability in both sequencing levels of nucleoids or even at the chromosomal level (7). Mutation intersect with these pathways will leads to transient phenomena because they include changes in repeated sequences which are hot spots for mutations or changes in the bands of chromosomes (8). Chemoprevention is defined as the use of natural dietary compounds and \ or synthetic substances that can delay , prevent or even reverse the development of adenoma as well as the progression from adenoma to carcinoma(9). The molecular mechanism of their chemo preventive action are associated with the modulation of signaling cascades , gene expression involved in the regulation of cell proliferation, differentiation, and apoptosis and the suppression of chronic inflammation, metastasis, and angiogenesis(11) .Prevention is the main strategy in order to reduce colorectal cancer incidence and mortality. It can be accomplished through primary prevention, using measures affecting factors known to confer higher risk of colorectal cancer, or through secondary prevention, aimed at early diagnosis of cancer or preneoplastic lesions in groups of subjects at increased risk of cancer(12)., at present it seems that secondary prevention is more effective on colorectal cancer survival, and the approaches which have yielded the most satisfying results, in terms of reduced mortality for cancer, are those aimed at detecting preneoplastic lesions, or cancer at an early stage in selected groups of subjects at average or increased risk of colorectal cancer(13). Medical reports have shown that chromosomal abnormalities and microsatellite early in the development of the tumor support the hypothesis of phenotype hypothesis, which shows that there are multiple pathways in maintaining gene stability in both sequencing levels of nucleoids or even at the chromosome level, mutations Which intersect with these pathways will lead to transient phenomena because they include changes in repeated sequences, which are hot spots for mutations or include changes in the bands of chromosomes (14).The aim of study is to investigate the cytogenetic parameters in order to define the effect of methotrexate (MTX) on blood lymphocytes of colon cancer patients in

comparison with healthy individuals, manifested by blast index (BI) , mitotic index (MI), replicative index (RI) , and sister chromatid exchanges (SCEs).

Materials and Methods:

Sample collection:

A total of 53 samples of colon cancer were collected from patientstreated with drug and 24 blood samples of healthy individual as control group .They were of different ages and gender. Samples were collected from Baghdad governorate from Al Yarmouk Hospital and Kadhimiya Hospital. The blood samples were collected (about 10 ml) from the infected patients and healthy individuals collected in glass test tubes treated with heparin. A questionnaire was prepared containing information about the groups of patients under study.

Transplantation of blood cells and genetic effects of MTX onlymphocytes:

About 0.4 ml of heparin-treated blood from patients with colorectal cancer, as well as normal healthy blood cultured in suitable media known as RPMI 1640medium supplemented with (10%) of bovine calf serum and 100 I.U of penicillin and 100 mg of streptomycin and 10 µg / ml of 5-Brduand 0.2 mL of PHA(phytohaemagglutinin) then treated with certain concentrations of methotrexate (concentrations (0.2, 0.5, 1, 2, 4, 8, µg / ml) and incubated at 37 ° C for 72 hours . Colchicine was added at a concentration of 10 µg / mL 3 hours before harvest and then incubated with the same previous conditions with stirring at certain times.

Collection of incubated cells after incubation:

The Cells were harvested aftercentrifugation at 2500 rpm / min for 10 minutes. The cells were treated with 5 ml of the hypotonic solution 0.075M (KCL) , then the tubes were placed in a water bath for 30 minutes. After the cells were collected by centrifugation at 2500rpm / min, the suspension was discarding and the fixative solution was added to the cells (methanol and acetic acid by 3 :1,) added by 5 milliliters of fixer solution and then left in the refrigerator for an hour and then the tubes centrifuged with the same conditions. Repeated the process of washing with the fix solution three times or more then took the cells and deposed and dropped by a Pasteur pipette on glass slides for the examination by light microscope. The glass slide containing the cells were stained with Giemsa stain for 5-10 minutes and then washed with distilled water. It was left to dry and then examined with a light microscope. Thetotal of cell number in each glass slide examined is about 1000 cells (10).

Calculation of the mitotic index (MI):

Mitotic index =

$$\frac{\text{number of dividing cells}}{\text{Total number of cells}} \times 100$$

Calculation of Blast index (BI) :

The blast index was calculated in the blood lymphocytes by using the following equation:

Blastogenic index (BI) =

number of tumor cells × 100

Total number of cells

The total number of cells in each glass slide examined is about 1000 cells (10)

Cell cycle progression:

The cell progress cycle was calculated by examining cells with the fluorescent microscope after staining the cells with DAPI stain samples for 10 minutes, then washing with distilled water, leaving to dry. The glass slide was treated with droplets of DAPI buffer, Cover the glass slide and press a little, then examine with Fluorescence microscope , counting the number of cells in the first division and in second division and the number of cells in the third division (10).

Replicative index (RI):

According to the number of dividing cells in the first division M1 and the number of dividing cells in the second division M2 and the number of dividing cells in the third division M3 and then Replicative index calculated as follows :

Replicative index RI =

$$\frac{M1 * 1 + M2 * 2 + M3 * 3}{100} \times 100$$

Sister Chromatid Exchange (SCEs):

The SCEs frequency were examined by stained with DAPI stain. They were then examined with the fluorescent microscope to detect the No. of SCEs e in each cell in the second division stage M2).

Calculate the mutant cells:

The number of mutant cells resistant to methotrexate was calculated in the following manner (Abbasid 2001).

No. of mutant cells =

No.of lymphocyte/ml multiplied by mitotic index 100

Results and Discussions:

The results in table (1) showed a significant decreases in the blast index with increases concentration of MTX in patient groups (colon cancer patients).The conc. Of MTX were (0.0,0.2 ,0.5, 1, 2, 4, 8) gave blast index that decrease with increase of MTX conc. It gave colon patients (28,6 , 18.6 , 14.5, 10.4 , 6.8 , 6.4 , 3.3) respectively. While in healthy gave (27.7 , 18.4, 17.01, 16.8, 12.3,8.6 , 5.3) respectively. PHA was used to stimulate lymphocytes in in vitro to study the vital events that occur after stimulation. The transformation of lymphocytes is a measure of the series of morphological and biological changes that occur in lymphocytes that are sensitive to antigens during the stages of division. The response of the lymphocytes to the

phytohaemagglutation depends on several factors, including the nature, number of (T cells) or (B cell cells) present in the medium, as well as the type and concentration of (PHA), where the interaction between specific antigens or PHA and lymphocytes leads to the transmission of signals Into the cell and activate the nucleus that is in a dormant state Go respond to the division and often accompanied by this response changes morphological into the lymphomas. This activity can be detected by measuring the blast index(11). The stages of cellular division begin with the G0 phase, which is usually the association of the antigen or the mitogen molecule by binding to a Ligand with a receptor on the surface of the lymphatic cell leading to the stimulation of the organisms responsible for cell activity. This is why the morphological changes appear as a result of the transformation of the rest cells when exposed to the mitogen to the blast cells where the size of lymphocytes increased and activate the cytoplasm and increase the number of vacuolesand become the nucleus visible inside the nucleus as a result of the combination of proteins of the nucleus. The biological changes increase membrane permeability, increase the flow of positive ions into cells and enter cells in the first growth phase, G1-phase, which is characterized by the production of proteins necessary for the cell and RNA and lymphocytes and then transformed cells into the S-phase and finally enter the sensitive cells to mitosis.

Table (1): Effect of different concentrations of methotrexate in the rate of Blastogenic Index (BI) in patients with colon cancer relative to the healthy

Blastogenic Index (BI) Mean ± SE		MTX)con.
Colon cancer patients	Healthy individuals	µg\ml
28.63 ± 4.56 Aa	27.73±2.5Aa	0
18.65 ± 3.2 Bb	18.40 ± 1.85Bb	0.2
14.50 ± 1.5 Bc	17.01±.042 Bb	0.5
10.43±1.3 Cd	16.18± 1.9 Bc	1
6.86± 0.85 De	12.28±1.40 Cd	2
6.72± 0.94Ee	8.65±1.01Ee	4
3.32± 0.60Ef	5.31±0.73 Ef	8

The values represent the mean of the cell division ± standard error. The different small letters indicate significant differences between the concentrations of the single transaction at the probability level $P\leq 0.01$ & $P\leq 0.05$ .The different letters indicate significant differences within each treatments (patient totals) at the probability level of ($P \leq 0.01$ & $P\leq 0.05$).

Effect of methotrexate in the rate of Mitotic Index(MI):

The effect of the drug on the rate of cell division was studied on 20 blood samples taken from healthy individuals and 53 blood samples taken frompatients with colon cancer. Different concentrations of methotrexate were used, starting with a zero concentration and then (0.2, 0.5-0, 1, 2, 4, 8 µg / ml). The results in table (2) presented a decrease in the rate of cell division by increasing the concentration of the drug in both showed the healthy and the patients individuals. The decrease was significant at the probability levelof P ≤ 0.01. In the control cells that represent the healthy, In the conc.of MTX(0.0,0.2,0.5,01,2,4,6) MI was (3.2% ,1.96 % , 1.57 %, 0.27% ,0.0,0.0 , 0.0) . The higher concentrations of the drug did not notice a division of cells. The statistical analysis represent the rate of the cell division and the standard error. Statistical analysis for healthy patients showed significant differences between zero concentration and other concentrations used in the research as shown in table2. In colorectal cancer,showed significant differences between zero concentration and other concentrations ,but no significant differences were found between the high concentrations of (1, 2, 4, 8, µg / ml). These results indicate that high concentrations of methotrexate have a toxic effect inblood lymphocyte cells of both healthy and colon cancer groups which also noticed with(12).

Table (2): Effect of Different Methotrexate Concentrations (MTX) on mitotic index (MI) in Colon Cancer Patients Compared to Healthy individuals

Mitotic index (MI) Mean ± SE		Drug Con.
Colon cancer patients	**Healthy individuals**	**µ g/ml**
3.63 ± 0.46Aa	3.2 ± 0.29Aa	0
2.31 ± 0.19 Bb	1.96 ±0.16 Bb	0.2
1.61 ± 0.32Bc	1.57 ± 0.17 Bb	0.5
0.18 ± 0.1Bd	0.27 ± 0.08Bc	1
0.22± 0.09Ad	0.0 ±0.0 Ac	2
0.0 ±0.0Ad	0.0 ±0.0Ac	4
0.0 ±0.0Ad	0.0 ±0.0Ac	8

The values represent the mean of the cell division ± standard error. The different small letters indicate significant differences between the concentrations of the single transaction at the probability level P≤0 .01 & P≤0.05 .The different letters indicate significant differences within each treatments (patient totals) at the probability level of (P ≤0.01 & P≤ 0.05).

Effect of Methotrexate in Replicative Index (RI):

The results in table (3) showed the effect of the drug in the rate of the replicative index in patients with colon cancer compared to the healthy individuals. It was noticed that increasing the concentration of the drug leads to a decrease in the(RI) significantly in both healthy and patients . The MTX conc.used (0.0 0.2, 0.5 ,1,2,4,8, µg / ml) It gave in the healthy individuals (0.85% ,0.35% , 0.30%, 0.06%)respectively .No cell division was shown in the high concentrations of (2, 4, 8 µg / ml). respectively. As well as with patient groups (colon cancer) also have the same effect in the same MTX conc. It gave (0.72% , 0.44%, 0.27%, 0.02%) respectively .This decrease indicates that the cells were unable to cross from the second division to the third division or perhaps the presence of a few cells that have been able to resist the concentration of the drug used, which is characterized as a drug against cellular metabolism if it works to stabilize the building of DNA by preventing the formation of purine and pyrimidine involved in the synthesis of DNA (13). This decrease is a significant decrease in the effect showed the drug has a toxic effect of the drug .It can be conclude that there is significant effect of the different concentrations of the drug on the replication index rate of the of patients compared to healthy people.

Table (3): Effect of Different Methotrexate Concentrations (MTX))on Replicative Index (RI) in Colon Cancer Patients Compared to Healthy individuals

Replicative Index (RI) Mean ± SE		(Drug Con.)
Colon cancer patients	Healthy individuals	µg/ml
0.72 ± 0.08 Aa	0.85 ± 0.13Aa	0.0
0.44 ± 0.04 Bb	0.38 ± 0.04 Bb	0.2
0.27 ± 0.06 Ac	0.3 ± 0.04 Ac	0.5
0.02 ± 0.005 Ad	0.06 ± 0.01 A d	1

Values represent rates for the cell cycleprogression ± standard error. The different small letters indicate significant differences within the single column between the concentrations of the single treatment at $P \leq 0.05$. The differentlarge letters indicate significant differences within the single row between the treatments of the single concentration at $P \leq 0.05$.

Effect of Methotrexate on induction of sister chromatid exchange (SCEs):

The data in table (4) showed that the rate of SCEs in healthy individuals in MTX conc. (0.0, 0.2 , 0.5µg / mL) gave (5.45 , 5.66 , 6.56) respectively , did not differ significantly at concentrations (0.2 and 0.5 µg / mL) with the values of SCEs compared to its control .In colorectal cancerpatients,

the chromatid exchange rate increased to 10.4 and 10.49 at the concentrations (0.2 and 0.5 µg / ml), respectively, compared with 8.47 in its control .The SCEs in colon cancer patients differed significantly from the healthy, treated group. This means an increase in the frequency of the chromosomal exchange rate (SCEs), which indicates mutations at the chromosomal level(15).

We conclude from Table (4) that the treatment of cells with concentrations (0.2 and 0.5 µ g / ml) of methotrexate has increased the frequency of SCEs due to increased damage in DNA and have the potential to induce genetic mutations this resuls agreed with (15)(16). In the concentration (1 µg / mL)for both the healthy and the colon cancer patients, SCEs can not be measured because the number of dividing cells was very small that can not be considered . SCEs isone of the genetic changes ,which helps to detect many of the antimicrobials and carcinogens in the cells, either directly or after activation, especially at very low concentrations of the substance. SCE frequency is affected by substances that affect the cell cycle, and produces a change in frequency of SCEs(17). These changes may not be related to the factor influencing the genetic material itself but to a change in the duration of cell multiplication. Several chemicals have been studied for their effects on the increases of SCEs such as mycomycin-C (MMC), cyclophosphamide, as well as aflatoxin B1 and caffeine(18) The use of methotrexate may also be considered as another measure of genetic analysis and represents a fracture in two double helices of DNA, where the chromosome strands are opened to the chromosome itself and then re-integrated into new sites(18) . That the re-join occurs inaccurately due to the failure of the system of DNA repair and that this may cause a change in the genetic codes, leading mating of the wrong (misspairing) and as a result may be deleted or implicated new rules leading to a breakthrough and reveals the exchange of chromatid brother as reciprocal sites of highly pigmented regions in the cellular chromosomes with the presence of Brdu (11) (18). This phenomenon is a sensitive indicator that can be used to estimate the extent of mutagenicity and carcinogenesis in laboratory systems. SCEs is more sensitive than micro nuclei and chromosomal abnormalities in the determination of genetic damage as a result of damage to the DNA molecule and thus increases the rate of chromatid exchange (19).

Elevated SCEs may be associated with disease severity. The mechanism by which cells can grow in these concentrations of the drug in the study, may be an amino acid replacement is shown at the protein site and as a result an increase in the effectiveness of the regulation lead to cell resistance to the drug. The presence of parietal proteins called p-glycoprotein, which have the potential to bind to the drug and thus prevent entry into the cell.(20). Another mechanism is the amplification of the gene responsible for DHFR, which is one of the main mechanisms of resistance. This means increasing the gene copies in a single cell (21). These results were consistent with those obtained by (11)in his study on cellular and enzymatic genetics in patients with colon cancer. As well as with AL-Amiry, 1999) in his study on lymphocytes of breast cancer.

Table (4): Effect of different concentrations of methotrexate in induction of sister chromatid exchange in colon cancer patients and healthy individuals

SCEs Mean ± SE		Drug Con.
Colon cancer patients	Healthy individuals	MTX conc.µg/ml
± 0.56 Ab8.47	5.45±0.45 Ba	0
10.41 ±0.88 Ba	5.66 ± 0.36 Ca	0.2
10.49 ± 0.14 Ba	6.56 ± 0.51 Ca	0.5
–	–	1

The values represent the average rates of SCEs ± standard error. Small letters indicate that there were significant differences within one column (between concentrations) within a single treatment at the probability level (P≤0.05) . Different large letters indicate significant differences within each treatments at P ≤0.05 between patients and healthy indinviduals.

Mutation frequency in blood lymphocytes of colon cancer patients compared with healthy individuals:

The results of table (5) represented the the mutation frequency and noticed mutant cells in colon cancer patients, for the high rates compared to the healthy individuals . In the healthy mutant cells were observed but a small percentage compared to the other groups as noted The rate of the mutation frequency in the concentration of (0.2 µg / mL) was 3.178 more than the mutation rate at 0.5 µg / mL, which was 2.17% and later decreased at 1 µg / mL, The statistical analysis in the healthy group showed no significant differences between the two concentrations (0.2, 0.5 µg / ml) at P ≤ 0.05, while significant differences were found in high concentrations (1, 2 and 4 µg / ml). In the first three concentrations ,the cells found to be resistant to the drug, while in the high concentration (2 µg / ml) it was observed to be cytotoxic and was subsequently followed by other high concentrations where no resistant cells were present. In patients with colon cancer, the statistical analysis showed no significant differences between the concentrations used for the same group of patients.

As for the effect of the drug on the mutation frequency coefficient between the groups, the results showed significant differences in the methotrexate concentrations between the healthy and grouppatients. More resistance was observed for the cells taken from colon cancer patients compared to the control .The readiness of groups of patients relative to the healthy. These mutant cells are likely to have to be transformed into cancerous cells. It was also observed that the resistance cells are often in the few concentrations in calculation of mutant cells and also depends on the number of lymphocytes in blood samples taken from patients and control groups , as these high concentrations are toxic concentrations, while the few

concentrations can induce the mutation in cells The results showed an inverse relationship between mutations and concentrations of methotrexate, which were agreed with those previously obtained (12)(11) .The study of cell resistance to the drug in addition to its importance as a chemical treatment, it gives an important benefit in the field of cellular genetics,(13)(14) because its importance in considering similar to the substance of the folate, which is important in metabolic processes, and the acquisition of mutations resistant to the drug gives information about the organization of metabolic events, Synthetic genes(22). The presence of mutant cells carrying the resistance to the methotrexate means a mutation in the encoded gene to DHFR Dihydrofolatereductase)(23).

Table (5): Mutation frequency in blood lymphocytes of colon cancer patients compared with healthy individuals treated with different Con. Of MTX

Colon cancer patients	Healthy individuals	MTX conc.µg/ml
		0
9± 5.77 Ab	3.17 ± 0.77 Aa	0.2
15.72±5.8Aa	2.17±0.42 Ab	0.5
4.1±2.86 Ac	0.74±0.46 Bb	1
0.0 ±0.0Ac	0.0 ±0.0 Bc	2
0.0 ±0.0Bc	0.0 ±0.0 Bc	4
0.0 ±0.0Bc	0.0 ±0.0 Bc	8

The values represent the mean of the cell division ± standard error. The different small letters indicate significant differences between the concentrations of the single transaction at the probability level P≤0 .01 & P≤0.05 .The different letters indicate significant differences within each treatments (patient totals) at the probability level of (P ≤0.01 & P≤ 0.05).

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