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The role of uracil-dna glycosylase and folate in the repair of dna

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THE ROLE OF URACIL-DNA GLYCOSYLASE AND FOLATE IN THE REPAIR OF DNA DAMAGE

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DEDICATION

I would like to dedicate my work to my loving and supportive parents; Talal and Elham; my caring siblings; Farah and Najib; my kind and encouraging family.

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I would like to thank my advisor Dr. Diane Cabelof, you have been a great support and mentor. Thank you for your patience and encouragement. I also want to acknowledge my committee members; Dr. Heydari and Dr. Zhou; for their help and support. Finally I would like to thank my friends and lab mates; Kirk, Aqila, Hongzhi, and Rita for their everyday help and encouragement.

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Introduction

DNA damage and Oxidative stress

DNA damage originates from endogenous as well as exogenous sources. UV light and radiation are among the main sources of exogenous DNA damage. Reactive oxygen species (ROS), which are produced through oxygen dis-metabolism, are the major source of the endogenous damage. Furthermore; endogenous damage can result from the instability of the chemical bonds in DNA, endogenous DNA methylating agents, or from DNA replication errors.

Endogenous DNA damage usually affects the primary structure of the DNA double helix. There are different types of modifications that result from endogenous damage; methylation is one of those modifications. Bases get methylated by endogenous molecules such as S-Adenosyl methionine (SAM). Methylation of guanine to 7-methyl-guanine and adenine to 3-methyl adenine creates lesions; the latter is more harmful due to its effect on the DNA replication process (13).

Endogenous damage can also cause hydrolysis of the bases; the following are the different ways through which the DNA damage is produced:

- Loss of bases in DNA due to the hydrolysis of the weak base sugar bonds
- Loss of the amino group in the 5-meC and the C resulting in the formation of the T and U bases that pair with the dAMP during replication, and lead to the rise of C to T, or C to A transitions.

Furthermore, oxidation, a major modification induced by endogenous DNA damage, is primarily caused by the byproducts of oxygen metabolism. Oxidation has been studied extensively, and it has been found that oxidation negatively impacts the genomic integrity through the formation of oxidized bases, such as 8-oxoG, thymine glycol, and other oxidized bases (fig 1.1). 8-oxoG is most frequent and harmful among all oxidized bases; 7500 8-oxoG forms each day in mammalian cells, and it usually mispairs with Adenine (13).

Oxidation has been associated with the promotion of cancer and ageing. Reactive oxygen species, such as O_2, cause DNA damage by directly interacting with DNA. O_2 selectively attacks guanine producing 8-hydoxyGuanine, and thus forming a DNA lesion. In addition, lipid peroxidation is another source of DNA damage; carbonyl containing compounds which, are products of lipid peroxidation, results in the formation of exocyclic DNA adducts.

Mechanisms through which oxidative stress is induced

H_2O_2:

H_2O_2 is a chemical compound that is generated in the body. H_2O_2 is generated during the synthesis of the thyroid hormone in the thyroid epithelial cells. This compound is capable of causing damage to the DNA by releasing free radicals such as hydroxyl radical, a reaction that involves Fe^{3+}, this reaction is known as the Fenton reaction:

1) $\mathbf{Fe^{2+} + H_2O_2 ----> Fe^{3+} + .OH + OH^-}$

2) $\mathbf{Fe^{3+} + H_2O_2 ----> Fe^{2+} + .OOH + H^+}$

The damage caused by the hydroxyl radical can be classified into 5 major classes; oxidized bases, abasic sites, DNA-DNA intrastrand adducts, DNA strand breaks, and

DNA-protein cross-links. Low concentration of H_2O_2 induces cell programmed death, a process known as apoptosis, which is a protective mechanism developed by the cell to prevent tumorigenesis (19).

To protect itself from the serious side effects of H_2O_2, the body has developed a defense mechanism against oxidative stress by detoxifying the H_2O_2 and superoxides. There are a number of enzymes in the body involved in the process of detoxifying H_2O_2 (fig 1.2), such as Super Oxide Dismutase (SOD) specifically SOD3, an isoform of the SOD that acts in the lumen the site of H_2O_2 generation (5).

The impact of H_2O_2 on DNA stability has been extensively studied in the past. Research labs have shown that the primary damaging capabilities of H_2O_2 was mediated through the free hydroxyl radical (17). The extent of damage caused by H_2O_2 was compared to that caused by hydroxyl radicals produced by the ionizing radiation, and it was established that it is similar to a great extent, and that the degree of different types of damage has the same order with base destruction having the highest magnitude, followed by single strand breaks, then double strand breaks, and finally crosslinks. Furthermore, it was shown that the extent of the DNA damage caused by H_2O_2 is also dependent on the presence of cuprous, ferrous, and ferric ions which can bind to inner and outer sites of the DNA (17).

Methotrexate (MTX):

MTX is classified as an anti-metabolite drug, and it has been used to treat diseases such as rheumatoid arthritis, Crohn's disease, and multiple sclerosis. This compound was also used to treat different kinds of cancers (head, neck, colorectal and certain types of leukemia, and lymphoma). MTX induces DNA damage in cancer cells. As the DNA damage becomes overwhelming to the cell, cell cycle arrest will be triggered. MTX was

able to inhibit the cellular proliferation of colon cancer cells (HT29 cells); however, this inhibitory effect was reversed when 100μM of thymidine and hypoxamine (Hx) were administered to the cells at the same time (39). It was suggested that MTX inhibits HT29 cellular proliferation by dramatically reducing the intracellular levels of adenosine and methionine ($p<0.001$), which are required in the synthesis of the methyl donor SAM (39).

MTX targets more than one enzyme (fig 1.3), including dihydrofolate reductase, thymidylate synthase, and AICAR transformylase (15). Methotrexate's main function is blocking the activity of dihydrofolate reductase (DHFR), which is an enzyme responsible for the reduction of dihydrofolate to tetrahydrofolate (THF); thus dihydrofolate will accumulate in the cell. THF is a vital cofactor in the process of DNA, RNA, and protein biosynthesis. Hence, the inhibition of DHFR, which will lead to the depletion of the THF pool, will negatively impact DNA, RNA, and protein synthesis; these processes are folate dependent. The efficiency of methotrexate depends on the availability of MTX in its free form after completely blocking the activity of the DHFR enzyme. Very low concentrations of active DHFR required for it to carry on its metabolic activity, thus excess methotrexate is required to completely inhibit the activity of the DHFR. Furthermore, DHF can compete with methotrexate to bind to DHFR, and activates it; and thus provides another reason to validate the importance of having unbound methotrexate inside the cell.

Methotrexate can also indirectly inhibit thymidylate synthase (TS) activity by inhibiting DHFR which will reduce the level of the folate substrate 5, 10- methylene FH4 available in the cell. However, inhibition of TS can be reversed by the presence of low amounts of folic acids which will lead to the repletion of the substrate 5, 10-methylene FH4. Moreover, MTX polyglutamate is a potent inhibitor of AICAR transformylase, which

is an enzyme that catalyzes the conversion of 10-formyl FH4 to FH4 and formyl AICAR; this reaction is an essential process for purine synthesis, thus the inhibition of AICAR transformylase by the MTX polyglutamates negatively impacts purine synthesis. Another potent inhibitor of the AICAR transformylase is FH2 polyglutamate, which accumulates as a result of MTX accumulation inside the cell.

MTX is polyglutamated inside the cell, due to the enhanced ability of MTX polyglutamates to inhibit DHFR and its increased retention inside the cell; the more glutamates added to MTX, the longer the time it will spend inside the cell, and thus the more efficient it is. In addition, MTX polyglutamates are also effective due to their comparable ability to inhibit Folate-dependent enzymes as well as Dihydrofolate reductase. Furthermore, MTX can be metabolized to 7-OH-MTX or to 3.3 Diamino-2,4-N-10-methylpetroic acid (DAMPA). The latter is less efficient in inhibiting DHFR, therefore metabolizing MTX into DAMPA a mechanism developed in the cell to detoxify MTX.

DNA Damage Repair

The cell has developed different pathways to correct the DNA damage to prevent the accumulation of the DNA damage which can be detrimental to the cell.

DNA Repair Pathways

The following are 3 main pathways through which the cell can repair the damage (6):

- Reversal of the damage
- Excision of the damage

- Tolerance of the damage

The pathway through which most DNA repair happens is the excision repair pathway. The following are the 3 different classes of excision repair:

- Mismatch repair (MMR)
- Nucleotide Excision repair (NER)
- Base excision repair (BER)

Mismatch repair is the pathway responsible for the repairing of replication errors, or errors due to the insertion or deletion of a nucleotide. This mechanism is very accurate and effective due to its ability to recognize which of the two bases is the mismatched base and its ability to replace it (6).

The primary role of the Nucleotide excision repair pathway is to excise large distorting DNA adducts. The enzymes involved in this pathway have a broad specificity, unlike the enzymes involved in BER and MMR (6).

BER is a major repair pathway for endogenous damage. It is the pathway responsible for repairing the small, non-helix distorting lesions that are a result of chronic oxidative stress (2), and correcting the spontaneous damages that arise as result of the ageing process (2). BER is activated to repair the mis-incorporated uracil. The following are kinds of damages repaired by BER pathway:

- Alkylated bases
- Oxidized bases
- Deaminated bases
- Bases with open rings
- Apurinic/apyrimidinic sites

BER is essential for the preservation of genomic and chromosomal stability by protecting the cells from mutational responses to carcinogens and DNA strand breaks. There are a number of enzymes involved in the base excision (fig 1.4) repair pathway. The first enzyme to initiate the repair pathway is the DNA glycosylase, which is responsible for the excision of the mis-incorporated base and the initiation of the BER pathway. There are two kinds of DNA glycosylases; the monofunctional and the bifunctional DNA glycosylases. The monofunctional glycosylases only have the glycosylase activity that enables them to excise the misincorporated base, whereas the bifunctional DNA glycosylases possess the AP lyase activity, and thus they are capable of cutting the phosphodiester bond of the DNA, which creates the single strand break without the need for the AP endonuclease activity. Table 1.1 includes examples of the human monofunctional and bifunctional DNA glycosylases involved in the BER pathway, and their possible substrates.

Uracil is excised by UDG; as well as other DNA glycosylases such as TDG, SMUG1, MBD4, NTH1, NEIL1, and NEIL2 which lead to the formation of a transient abasic site. The AP endonuclease (APE) will then create a transient 3'OH inducing a single strand break and a deoxyribose phosphate flap. This flap will be removed in the following step by means of β-pol, which is also responsible for the insertion of the correct nucleotide. The last step is the ligation of the strand where the single strand break was created; this step is carried out by XRCC-1/ligaseIII .

Folate and its role in DNA repair

Folate is one of the most important micronutrients due to the role it plays in DNA metabolism; it is involved in the process of uracil conversion to thymine. Furthermore, folate is vital for the prevention of certain birth defects (including Spina bifida), prevention

of anemia, and formation of red blood cells. In addition, folate along with vitamin C and vitamin B12 help in the breakdown, utilization, and formation of proteins.

Folate is an essential factor to maintain DNA and genomic integrity due to its involvement in the following processes:

- **DNA methylation**

There are 2 different types of methylation; DNA methylation that regulates gene expression, and histone methylation that determines the chromosomal conformation. Folate is required for the synthesis of the S-Adenosyl methionine (SAM) a methyl donor required for DNA methylation. In the absence of folate, methionine remythelyation is reduced which leads to a reduction in SAM synthesis; as a result the ability to methylate DNA will be negatively impacted.

- **DNA repair and synthesis**

DNA repair and synthesis is essential to maintain the integrity of the genome. Methylenetertrahydrofolate reductase (MTHFR) is usually inhibited by SAM to stop the process of irreversible conversion of 5,10-tetrahydrofolate to 5-methyltetrahydrofolate, therefore preventing the formation of Uracil in excess and its incorporation into the DNA; the inhibition of MTHFR by SAM is reduced in case of folate deficiency (9).

- **Thymidylate synthesis**

Thymidylate synthesis is the process required for the synthesis of thymidine monophosphate (dTMP) and then phosphorylated to thymidine triphosphate (dTTP) that gets incorporated into DNA. The enzyme thymidylate synthase (TS) is the enzyme involved in the catalysis of the reaction that involves the transfer of one methyl group from

5,10- MeTHF- a byproduct of folate metabolism- to dUTP in order to generate dTTP (fig 1.5).

Folate deficiency has been associated with cancer development. The carcinogenic properties of folate deficiency has been tied to a number of factors including a reduction in the levels of SAM (43), depletion of thymidylate, and reduction in the biosynthesis of purines (44). Folate deficiency has been experimentally demonstrated to be correlated to the development of liver and colon cancer by increasing the carcinogenic effect of dimethylhydrazine, a potent carcinogen that acts as a DNA alkylating agent used to induce colon cancer in experimental models (45). Furthermore, it was found that folate/methyl deficient rats had accumulated preneoplastic changes in their liver (46).

Folate deficiency has also been associated uracil accumulation into DNA. It has been shown that 4 million uracils are mis-incorporated into the DNA of folate deficient individuals (48). Uracil accumulation results in point mutations, single strand breaks, and double strand breaks, along with the risk of micronucleus formation and chromosomal breakage (33 and 35), which increases the risk of cancer development.

In vitro studies have shown that culturing lymphocytes in the absence Folic acid and thymidine in the culture media, lead to the expression of fragile chromosomes, micronucleus expression, and chromosome breakage (9). Furthermore, it has been observed that lymphocytes cultured with low concentrations of folic acid (15-30nM) have an apoptotic response; this response is reflected by a statistically significant (two fold increase) in p-53 compared to lymphocytes cultured with 120nM folic acid (25). However, lymphocytes cultured with 15nM folic acid developed DNA strand breaks in the p-53 DNA

sequence, revealed in a statistically significant reduction of amplifiable DNA exons 5-8 and 7-8 of the p-53 sequence (25).

DNA damaging effects of folate deficiency are more detrimental than that of high doses of ionizing radiation (23). It has been shown that physiologically relevant levels of folate deficiency was found to have more significant effect than exposure to high doses of ionizing radiation on the growth inhibition of primary human lymphocytes, induction of apoptosis, generation of DNA breaks (both double and single stranded breaks), induction of differential gene expression changes, and the cessation of lymphocyte cell cycle (23).

UNG and its role in DNA repair

UNG is the gene that codes for the enzyme Uracil-DNA Glycosylase (UDG). UDG is a vital protein in the DNA repair pathway that removes the misincorporated uracil from the DNA strand creating an abasic site. UNG has two different isoforms, the mitochondrial form UNG1 and the nuclear form UNG2, with UNG2 being the dominant form in cells (representing more than 90% of the enzyme's activity.

Usually uracil incorporated into the DNA is removed during the S-phase, which suggests that UDG is mainly active during the S-phase. UDG interacts with PCNA (proliferating cell nuclear antigen) and RPA (replication protein A) (12); these are two of the proteins that form the functional replication fork, and they are found in the replication foci. Furthermore, UDG interacts with the CENP-A (human centromere protein A), whose main function is to separate the centromeres during mitosis (12), which suggests that UDG is involved in the process of cellular proliferation.

The removal of uracil leads to the formation of abasic sites, which are more toxic to the cell than the uracil that is removed from the DNA (32). However, it was suggested that UDG reduces the toxicity of the abasic sites by binding to these sites until it's been

removed by the AP endonucleases (12). Moreover, it was observed that UDG plays an essential role in the prevention of GC to AT transition mutation (32).

An overexpression of ung1 in fission yeast, which has a similar function as the nuclear isoform UNG 2 in humans, was shown to delay the cell cycle by activating checkpoints.

According to a recent study, it was documented that the cells (DLD 1 colon cancer cells) sensitivity towards drugs such as, pemetrexed, Cisplatin, TMZ, and 5-fluorouracil, which are drugs that induce DNA damage, is affected when Ung is knocked out; Ung deficient cells are 10 times more sensitive towards pemetrexed compared to that of the wild type cells (12).

Moreover, pemetrexed exposure was found to have an impact on cell cycle progression between $Ung^{+/+}$ and $Ung^{-/-}$ cells, where the S phase was stalled in $Ung^{-/-}$ cells and the expression of the checkpoints proteins; such as phospho Chk1 (Ser345), phosphor cdc2, and cyclin B1; were induced to a greater extent in $Ung^{-/-}$ cells, which indicates a further induction of the S and M- phase checkpoints in $Ung^{-/-}$ cells (32).

Additionally, an induction in the expression of certain proteins; such as phosphor histone H3 (a mitotic marker), G1-S phase specific cyclin D1 and E1 protein; responsible for DNA damage checkpoints was detected following the exposure to pemetrexed.

Chapter 2

Hypothesis and Specific Aims

The impact of DNA damage and oxidative stress on genomic integrity has been widely investigated due to its association with cancer and ageing. DNA repair pathways play a critical role in preserving the genomic integrity and protecting the cells against DNA damage caused by oxidative stress. Base excision repair (BER) is one of the major pathways involved in the cellular response against oxidative stress and DNA damage.

Furthermore, the effect of folate deficiency has also been examined due to its impact on the cells ability to repair the DNA damage. Studies have demonstrated that Folate deficiency stalls the DNA repair ability of the cell by inducing Uracil accumulation and depleting the thymine pool, and reducing the capacity to methylate DNA. In addition, it has been established that folate deficiency fails to induce the BER response to oxidative stress by inhibiting β-pol expression at the transcriptional level (40).

The purpose of this project is to study the effect of genotype (the presence or absence of UNG) and folate deficiency on the cell viability and its response to oxidative stress and DNA damage induced by H2O2 and MTX. In addition, we want to examine the effect of genotype on the uracil accumulation in treated and untreated cells.

We hypothesize that UDG sensitizes cells to DNA damage induced by H2O2 and MTX. We are going to test this hypothesis by measuring cell viability in response to genotype and exposure. In addition, we are going to quantify Uracil misincorporation in

H2O2/MTX treated cells and untreated cells. I plan to test the hypothesis with the following specific aims:

Specific Aim 1: To study the impact of folate depletion on cell growth

Specific Aim 2: To study the impact of genotype ($Ung^{+/+}$ vs $Ung^{-/-}$) on the cell viability in response to DNA damage induced by H_2O_2 and MTX.

Specific Aim 3: To study the impact of folate on the cell viability in response to DNA damage induced by H_2O_2 and MTX.

Specific Aim 4: To study the impact of folate, genotype ($Ung^{+/+}$ or $Ung^{-/-}$), and MTX on the uracil accumulation in cells

Chapter 3

Materials and Methods

Tissue culture:

Mouse embryonic fibroblasts cell line (MEFs); two types of MEF cells were used in this project, wild type for $UNG^{+/+}$ and the knock out $UNG^{-/-}$. The MEF cells are immortalized when the SV-40 Tag (binding activity attributed to the large T-antigen) which binds to the p53 protein (a tumor suppressor that induces cellular apoptosis), thus disabling the protein.

The MEF cells were also grown under different conditions; folate added and folate depleted DMEM media. The complete media is made up of 10% FBS (the FBS is dialyzed in the 0% folate media), 1% glutamax, 0.5% glutamine, and 1% antibiotic. The cells are grown in an incubator at 37ºC and 5% CO_2.

MEF's were grown in Folate-free DMEM media and supplemented with dialyzed FBS. MEF's were supplemented with Adenosine and Thymine at different concentrations in the absence of Folic Acid. Adenosine/Thymidine were depleted in a step-wise manner starting with a 1X concentration. The cells were grown for 3 passages at each Ad/thy concentration and grown to 75% confluency before each passage. The cells were passaged down until 0% Adenosine/thymidine was achieved.

Folate microbiological assay

Lactobacillus casei microbiological assay to detect the folate level in the cells; we have two groups of cells, the folate replete and folate deplete, and each group has 4 samples. The method is a modification of Donald's. L. casei are grown overnight in the growth media with folic acid. The plate is setup by adding 8µl of the working buffer (3.2g sodium ascorbate + 19ml water + 1ml 1M potassium phosphate buffer PH 6.1), 150µl of the single strength folic acid casei medium, the sample (1µl or more depends on the sample's concentration), then distilled water is added to adjust the total volume to 180ul. Finally, add 20µl diluted L. caser inoculums to each well. The plate is then covered with polystyrene cover and aluminum foil, and incubated at 37˚C for about 21hrs, and read at 595nm in the model Genios basic of TECAN-GENios plus plate reader with the software TECAN megellan v6.00. The results were analyzed using the t-test ($p< 0.05$).

Cell Titer Blue Viability assay

We used the cell titer blue dye (Resazurin,Promega), which identify viable cells through detecting the metabolic capacity of these cells, as only viable cells are capable of reducing the Resazurin to Resorufin, which is the substance that fluoresces. We used a plate reader that detects the fluorescence emitted by Resorufin, and estimates the number of viable cells based on the fluorescence intensity. The emission and excitation wavelength used were 570nm and 635nm respectively. The plate is read 4 hours after the Cell Titer Blue dye is added.

On day 1 the cells are plated in a 96-well plate (8000 cells in each well). The total volume in each well is 200µl, including the volume of cells, the media, and the drug (MTX/H_2O_2); the concentration of the stock solution of H_2O_2 and MTX was 10mM and 50nM respectively. On day 2 (18 to 24 hours post-platting), the media is removed (using

the vacuum pump), wells are washed with 1XPBS, 200µl of fresh media is added to each well, and then the cell titer blue dye is added.

Doubling time

Cells were grown in the media with a starting number of 250,000 cells. The cells were incubated at 37°C and 10% CO2 until they were 75% confluent. Cells are then counted to get the cells number (N_1). We used the following formula to calculate the doubling time (Incubation time was calculated in hours (t)):

Doubling time = $t^* \ln(2)/\ln(N_1/N_0)$.

Genomic DNA isolation

TAg $Ung^{+/+}$ Cells were treated with 100nM of MTX or 100 µM H_2O_2 for 4 hours, and then both treated and un-treated cells were harvested. We used Qiagen's gravity tip columns and the protocol that came from the manufacturer to isolate the genomic DNA.

Uracil detection assay

The first step following the genomic DNA isolation is blocking the abasic sites with methoxamine (Mx, sigma) for 2 hours at 37ºC; genomic DNA is quantified, and equal amount of DNA is used (4µg of DNA brought up to 100µl in TE buffer. A working solution with equal volumes of tris/methoxamine is prepared; the final concentrations of tris and methoxamine are 50mM and 100mM respectively. DNA is then precipitated by adding 10% 2M NaCl, glycogen (final concentration of 0.4µg/µl), 1 volume of isopropanol, and 70% ice cold EtOH. The DNA is re-suspended in TE buffer.

In the second step, samples are treated with 0.2U of UDG for 15 minutes at 37ºC. DNA is precipitated as described above, and then re-suspended in TE buffer.

In the third step, the samples are treated with 2mM ARP probe (Dojindo) for 15 minutes at 37ºC. DNA is precipitated with isopropanol/EtOH and dissolved in TE buffer.

In the last step, the DNA (0.5µg) is heat denatured at 100ºC for 10 minutes. 2M ammonium acetate (220µl) is added to prevent the re-annealing of the DNA, and then each sample is loaded into a well of the slot blot apparatus, that has a nitrocellulose (NC) membrane (Schleicher & Schuell) inside (vacuum is applied). The NC membrane is washed with 5X SSC at 37ºC for 15 minutes, and baked in the oven at 80ºC for 30 minutes. 40 ml of hybrid mix buffer (1M tris, 5M NaCL, 0.5 EDTA, 10% Tween-20, 100mg BSA, and 200mg Casein) is added to the membrane and incubated for 30min at room temperature. Another 40 ml containing 20µl of concentrated HRP/Streptavidin (BioGenex) is added to the NC membrane and incubated for 45 minutes at room temperature. In the final phase of this experiment, the membrane is washed 4 times with 100 ml of TBS (0.5M EDTA, 1M tris, 10% Tween-20), and finally the membrane is incubated with ECL (Pierce Super Signal West Pico Chemiluminescent Substrate) reagent for 5 minutes at room temperature. The bands are visualized and quantified using the ECL/Chemilmager system

Statistical Analysis:

Statistical significance between the % of viable cells of the MEF cells with different UNG genotype and growth conditions (FA or FD medium) using the Graphpad Prism. $P < 0.05$ is considered to be statistically significant.

Chapter 4

Results

Folate measurements in folate added and folate depleted cells

The purpose of this experiment was to confirm that the process of folate depletion was successful by measuring the folate concentration. According to figure 4.1 the $UNG^{+/+}$ and $UNG^{-/-}$ folate depleted cells had significantly lower folate concentrations as compared to the $UNG^{+/+}$ and $UNG^{-/-}$ cells cultured in the presence of folate ($p<0.05$)

Impact of Folate Deficiency on the cellular proliferation

The purpose of this experiment was to determine the impact of folate deficiency on the cellular proliferation of $UNG^{+/+}$ and $UNG^{-/-}$. As shown in figure 4.2, the doubling time for the MEF cells cultured in absence of folate was significantly greater than that of MEF cells cultured in a complete medium. Moreover, there was no significant difference in the doubling times of $UNG^{+/+}$ and $UNG^{-/-}$ cultured in the presence of folate. Whereas, in a folate deficient media the doubling time of $UNG^{-/-}$ was significantly different than that $UNG^{+/+}$.

Cytotoxicity

The impact of UNG genotype on the response to H_2O_2 and MTX in FA medium

We studied the impact of UNG genotype on the ability of the cells to resist H_2O_2 treatment at different concentrations in a FA medium. As shown in figure 4.3A $UNG^{-/-}$ is

more resistant than $UNG^{+/+}$ MEF cells to H_2O_2 dosages higher than 20µM. However, in both cases cells were almost all dead at concentration equals to 100µM. EC50 values represent the concentration of treatment at which half the cells are still viable. The difference in the EC50 values (Fig4.3B) between $UNG^{+/+}$ (51.75µM) and $UNG^{-/-}$ (93.58µM) MEF cells was found to be statistically significant ($p<0.05$).

Moreover, we examined the impact of UNG genotype on the ability of the cells to resist MTX treatment at different concentrations in FA medium. As shown in figure 4.4A, both $UNG^{-/-}$ and $UNG^{+/+}$ MEF cells responded in the same manner to the MTX treatments of different concentrations. In both cases cells resisted MTX concentrations up to almost 16nM which was followed by a sharp decrease in cell viability until it reached zero at a concentration between 16nM and 32nM. The difference in the EC50 values (Fig4.4B) between $UNG^{+/+}$ (16.75nM) and $UNG^{-/-}$ (15.89nM) TAg cells was found to be statistically insignificant ($p>0.05$).

The impact of UNG genotype on the response to H_2O_2 and MTX in FD medium

We observed the impact of UNG genotype on the ability of the cells to resist H_2O_2 treatment at different concentrations in FD medium. As shown in figure 4.5A the cell viability of $UNG^{+/+}$ was continuously decreasing at low dosages of H_2O_2, whereas $UNG^{-/-}$ cells were able to resist H_2O_2 dosages less than 20µM with a rate of death slower than that of $UNG^{+/+}$; thus $UNG^{-/-}$ exhibit a less sensitive response to H_2O_2. However, in both cases cells were almost all dead at concentration equals to 100µM. The difference in the EC50 values (fig 4.5B) between $UNG^{+/+}$ (20.62µM) and $UNG^{-/-}$ (50.27µM) TAg cells was found to be statistically significant ($p<0.05$)

Moreover, we examined the impact of UNG genotype on the ability of the cells to resist MTX treatment at different concentrations in absence of folate. As shown in figure 4.6A the cell viability of $UNG^{+/+}$ was continuously decreasing at lower dosages of MTX, whereas $UNG^{-/-}$ cells were able to resist MTX dosages less than 16 nM with a rate of death slower than that of $UNG^{+/+}$. However, in both cases cells were all dead at 32nM MTX. The difference in the EC50 values (fig 4.6B) between $UNG^{+/+}$ (5.7nM) and $UNG^{-/-}$ (17.1nM) TAg cells was found to be statistically significant ($p<0.05$).

Impact of folate depletion and UNG genotype on the accumulation of Uracil

We observed the impact of folate depletion the impact on uracil accumulation in DNA. According to figure 4.8, folate deficiency induces uracil accumulation. We detected a statistically significant difference in the uracil accumulation in folate depleted $UNG^{-/-}$ cells compared to the $UNG^{+/+}$ folate depleted cells and the $UNG^{+/+}$ and $UNG^{-/-}$ folate added cells ($p<0.05$). Moreover, we detected a statistically significant difference in uracil accumulation in the $UNG^{+/+}$ cells cultured in folate depleted as opposed to $UNG^{+/+}$ cultured in folate added medium ($p<0.05$).

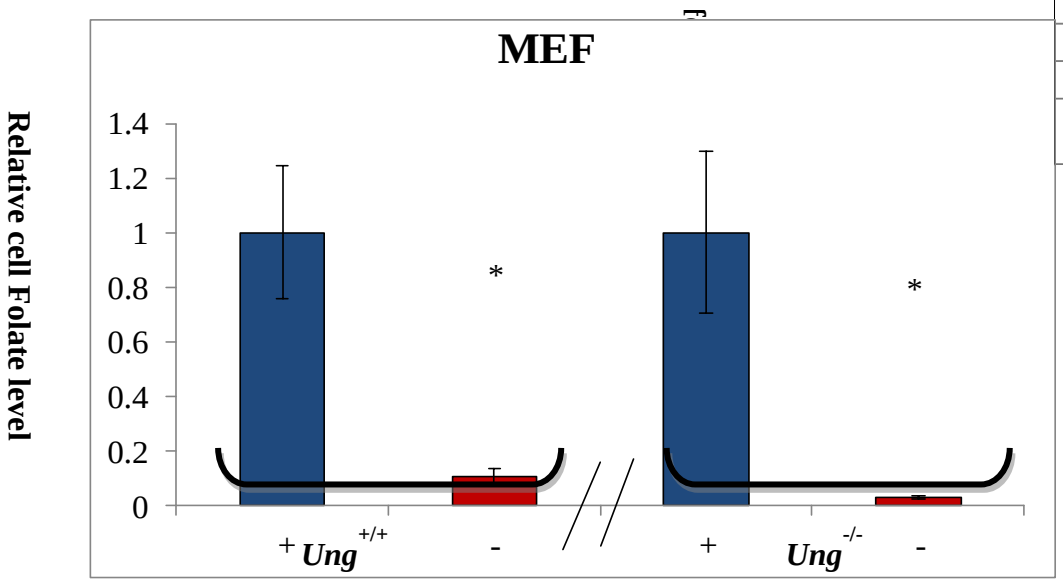
In addition, we studied the impact of UNG genotype on uracil accumulation. According to Figure 4.9, uracil accumulation was induced in $UNG^{-/-}$; we detected a 3 fold difference in the uracil concentration between $Ung^{+/+}$ and $Ung^{-/-}$ MEFs .

Impact of MTX on Uracil accumulation

We examined the impact of MTX treatment on the uracil accumulation in DNA. According to figure 4.10 MTX treatment induced uracil accumulation in

the treated cells compared to the control group; uracil accumulation in the treated group was induced to greater extent (by 3-folds) in the $Ung^{-/-}$ MEFs.

Figure 4.1



t test	p value	fold
207+/207-	0.023	9.7
92+/92-	0.033	35.6
207+/92+	0.466	N/A
207-/92-	0.053	4.7

Folate added (FA)
Folate deficient (FD)

Figure 4.1 Folate measurements in folate added and folate depleted cells

The lactobacillus casei assay was used to detect the folate level in the MEF cells. L.Casei were grown overnight in growth media with folate. A plate was setup up with the sample treated with the conjugase for 4 hrs, the single strength folic acid casei medium, and the working solution. After incubation for 21hrs the plate was read at 595nm with a plate reader (TECAN_GENios). The values represent the concentration of folate (fmol/cell) in each cell group. The difference between folate added and folate depleted $UNG^{+/+}$ or $UNG^{-/-}$ was statistically significant ($p<0.05$)

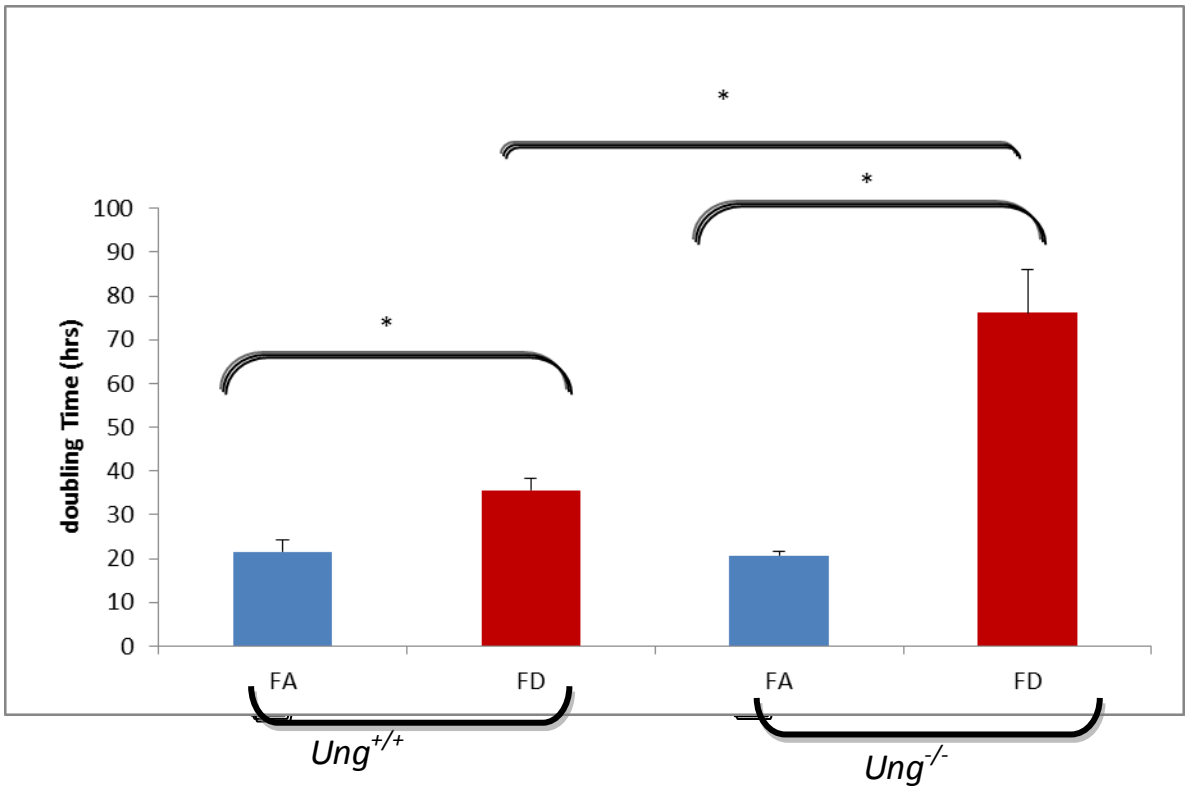
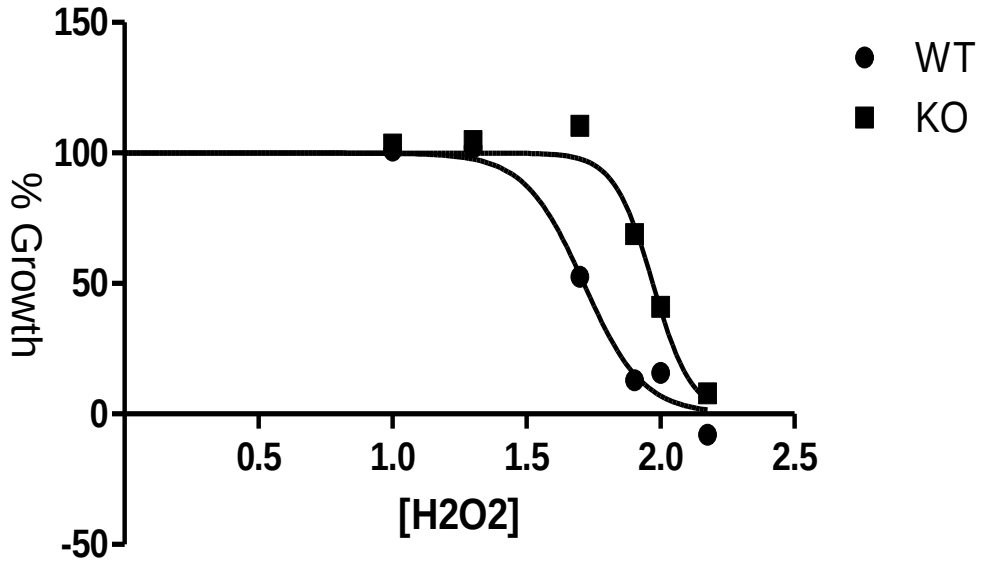

doubling Time (hrs)
100
90
80
70
60
50
40
30
20
10
0
*
*
*
FA
FD
FA
FD
Ung+/+
Ung-/-

Figure 4.2 Impact of Folate Deficiency on the Growth of $UNG^{+/+}$ and $UNG^{-/-}$ MEF cells. Cells were grown in complete and folate depleted media. Cells were counted when they reached 75% confluency and a formula was used to calculate the doubling time. The values above represent the doubling time in hours for each cell type. The difference in doubling time between the different groups was statistically significant ($p<0.05$)

Figure 4.3



(A)

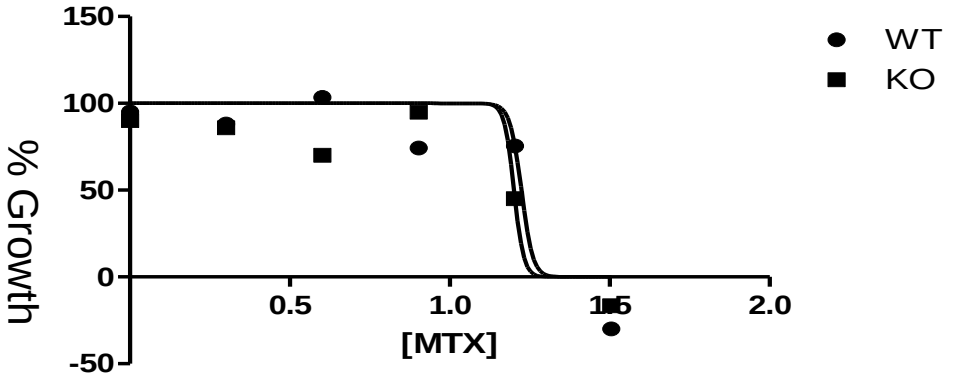
Cell Group	EC:50 WT	KO	p-value
FA cells treated with H2O2	**48.2**	**90.0**	**0.013**
FA cells treated with MTX	16.9	14.7	0.16
FD cells treated with H2O2	20.8	46.1	0.014
FD cells treated with MTX	6.5	17.5	0.022

(B)

Figure 4.3 The impact of UNG genotype on the response to H_2O_2 in a FA medium

Cells were platted in a 96-well plate with media and H_2O_2. The cell titer blue dye is added after 24 hrs. The plate is read 4hrs later using a plate reader at 570nm emission and 635nm excitation wavelength to determine the number of viable cells (A) the values represent the % of viable $UNG^{+/+}$ and $UNG^{-/-}$ cells treated with different H_2O_2 concentrations. (B) EC50 values for $UNG^{+/+}$ and $UNG^{-/-}$ that represents the dosage at which half of the cells are dead. The variation is statistically significant ($P<0.05$).

Figure 4.4



(A)

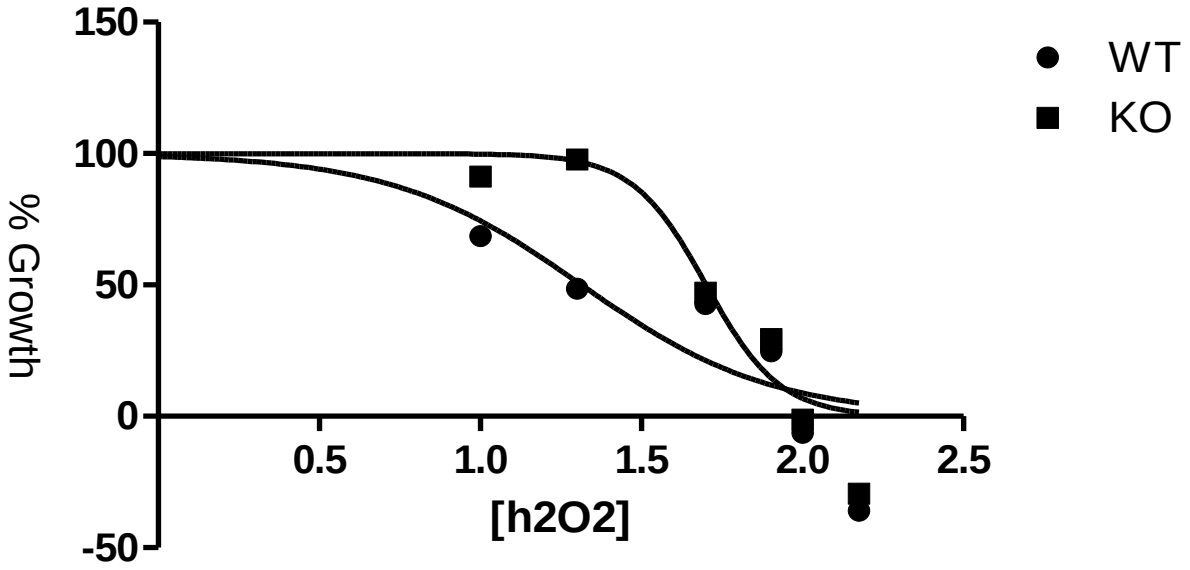
Cell Group	EC:50 WT	KO	p-value
FA cells treated with H2O2	48.2	90.0	0.013
FA cells treated with MTX	**16.9**	**14.7**	**0.16**
FD cells treated with H2O2	20.8	46.1	0.014
FD cells treated with MTX	6.5	17.5	0.022

(B)

Figure 4.4 The impact of UNG genotype on the response to MTX in FA medium

Cells are platted in a 96-well plate with media and MTX. The cell titer blue dye is added after 24 hrs, and then the plate is read 4hrs later using a plate reader at 570nm emission and 635nm excitation wavelength to determine the number of viable cells (A) the values represent the % of viable $UNG^{+/+}$ and $UNG^{-/-}$ cells treated with different MTX concentrations. (B) EC50 values for $UNG^{+/+}$ and $UNG^{-/-}$ that represents the dosage at which half the cells are dead. The variation is statistically insignificant ($P>0.05$)

Figure 4.5



(A)

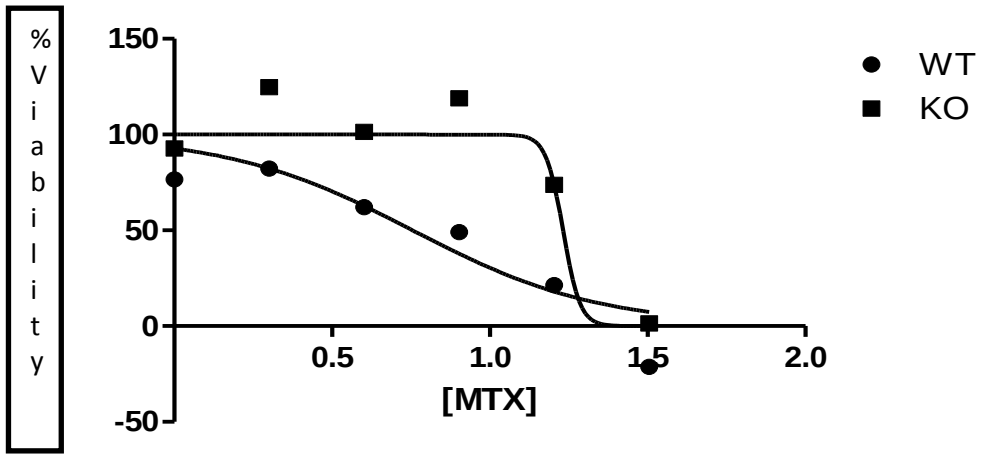
Cell Group	EC:50 WT	KO	p-value
FA cells treated with H2O2	48.2	90.0	0.013
FA cells treated with MTX	16.9	14.7	0.16
FD cells treated with H2O2	**20.8**	**46.1**	**0.014**
FD cells treated with MTX	6.5	17.5	0.022

(B)

Figure 4.5 The impact of UNG genotype on the response to H_2O_2 in FD medium

Cells are platted in a 96-well plate with folate free media and H_2O_2. The cell titer blue dye is added after 24 hrs. The plate is then read 4hrs later using a plate reader at 570nm emission and 635nm excitation wavelength to determine the number of viable cells (A) the values represent the % of viable $UNG^{+/+}$ and $UNG^{-/-}$ cells treated with different H_2O_2 concentrations in a Folate depleted medium. (B) EC50 values for $UNG^{+/+}$ and $UNG^{-/-}$ that represents the dosage at which half of the cells are dead. The variation is statistically significant ($P<0.05$)

Figure 4.6



(A)

		EC:50		
	Cell Group	WT	KO	p-value
	FA cells treated with H2O2	48.2	90.0	0.013
	FA cells treated with MTX	16.9	14.7	0.16
	FD cells treated with H2O2	20.8	46.1	0.014
→	**FD cells treated with MTX**	**6.5**	**17.5**	**0.022**

(B)

Figure 4.6 The impact of UNG genotype on the response to MTX in FD medium

Cells are platted in a 96-well plate with folate free media and MTX. The cell titer blue dye is added after 24 hrs, and then the plate is read 4hrs later using a plate reader at 570nm emission and 635nm excitation wavelength to determine the number of viable cells (A) the values represent the % of viable $UNG^{+/+}$ and $UNG^{-/-}$ cells treated with different MTX concentrations in a Folate depleted medium. The % of viable cells was determined using the cytotoxicity assay which provides us with a count of the living cells. (B) EC50 values for $UNG^{+/+}$ and $UNG^{-/-}$ that represent the dosage at which half of the cells are dead. The variation is statistically significant ($P<0.05$)

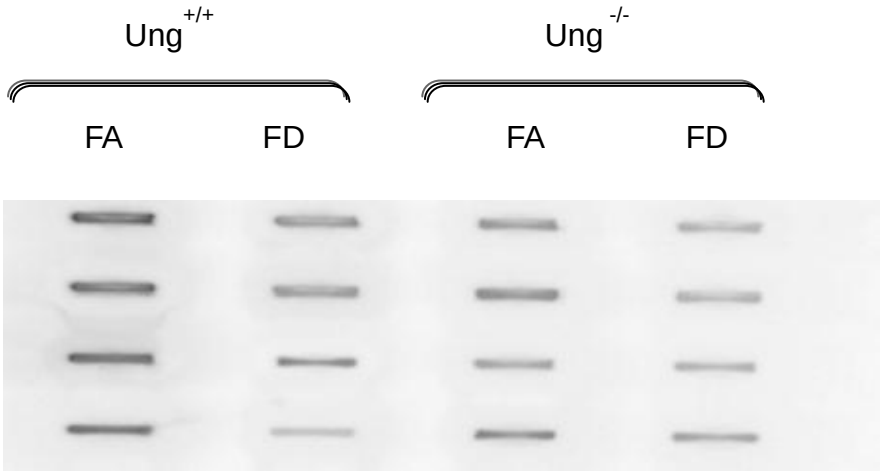
Figure 4.7

	EC:50		p-value
Cell Group	WT	KO	
FA cells treated with H2O2	48.2	90.0	0.013
FA cells treated with MTX	16.9	14.7	0.014
FD cells treated with H2O2	20.8	46.1	0.16
FD cells treated with MTX	6.5	17.5	0.022

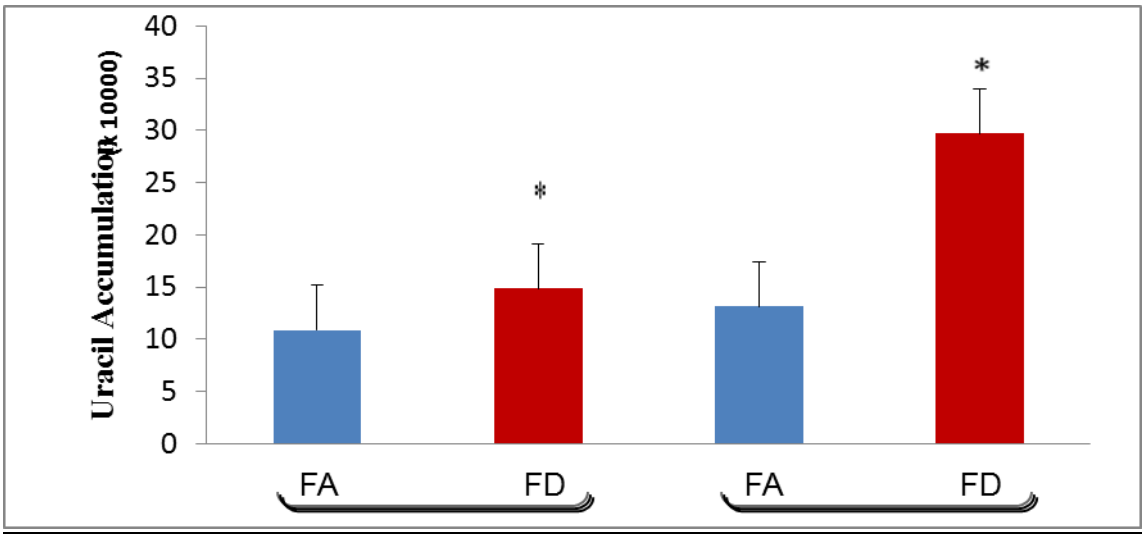
Figure 4.7 EC:50 values for H2O2 and MTX treated MEFs cultured in FA and FD medium

This table represents the EC:50 values of the different cell groups that was computed using the graphpad prism software.

Figure 4.8



(A)



(B)

Ung+/+ Ung-/-

Figure 4. 8 Impact of folate depletion on Uracil accumulation

Genomic DNA is isolated from $UNG^{+/+}$ and $UNG^{-/-}$ MEF cells cultured in folate added and folate depleted medium. DNA was blocked with methoxyamine, treated with UDG, and then probed with ARP. The samples were blotted on a nitrocellulose membrane and then bands were visualized by exposure to fluorescence. The bars represent the average uracil levels. The difference in the uracil levels was statistically significant ($p<0.05$)

Figure 4.9

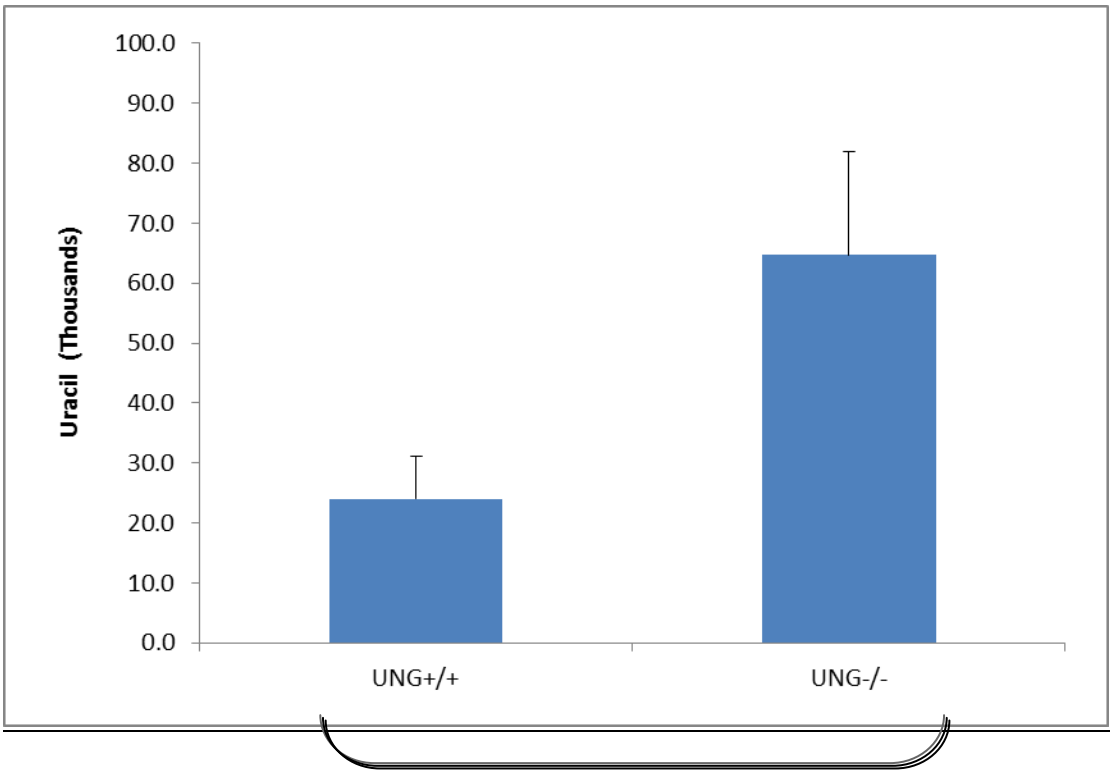
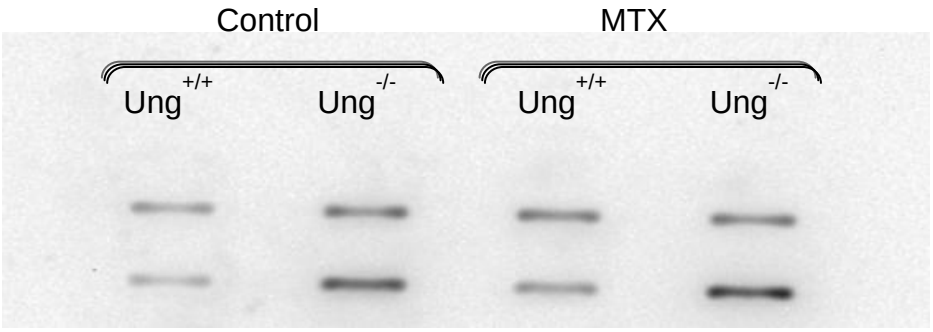
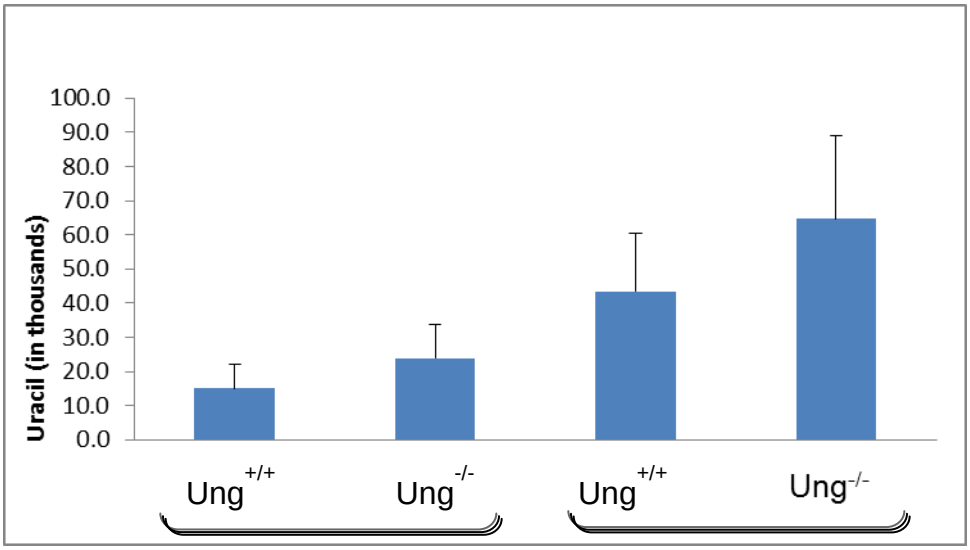

100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Uracil (Thousands)
UNG+/+
UNG-/-
FA

Figure 4.9 The impact of UNG genotype on the uracil accumulation

Genomic DNA is isolated from $UNG^{+/+}$ and $UNG^{-/-}$ MEF cells cultured in folate added medium. DNA was blocked with methoxyamine, treated with UDG, and then probed with ARP. The samples were blotted on a nitrocellulose membrane and then bands were visualized by exposure to UV fluorescence (BioRad). We detected an increase in uracil accumulation in the absence of *Ung* ($p>0.05$).


Control
MTX
Ung+/+
Ung-/-
Ung+/+
Ung-/-

(A)


Uracil (in thousands)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Ung+/+
Ung-/-
Ung+/+
Ung-/-

(B)

Figure 4.10 Impact MTX treatment on uracil accumulation

Genomic DNA was isolated from $UNG^{+/+}$ and $UNG^{-/-}$ MEF cells cultured in folate added medium. DNA was blocked with methoxyamine, treated with UDG, and then probed with ARP. The samples were blotted on a nitrocellulose membrane and then bands were visualized by exposure to fluorescence. The bars represent the average uracil accumulation in the cell. We detected the an increase in uracil accumulation as a result of MTX treatment; however, it wasn't statistically significant ($p>0.05$)

Chapter 5

Discussion

The protective role of uracil-DNA glycosylase to cytotoxicity and mutagenicity caused by oxidative stress and DNA damage, as well as the impact of UDG on the cell cycle progression, has been extensively studied. Opposing hypotheses and conflicting results have emerged regarding the role of UDG. In our study, we detected an increase in the uracil accumulation and the doubling time in the DNA of Ung null cells; this indicates that UDG induces uracil mis-incorporation and slows down the cell cycle progression.

Moreover, we observed a decreased viability of $UNG^{+/+}$ (as compared to $UNG^{-/-}$ cells) treated with H_2O_2 in a folate added and folate depleted medium; therefore UNG sensitizes cells to H_2O_2. On the other hand, the impact of UNG genotype on the cells response to MTX was associated with the folate status in the medium; UNG genotype had no impact on the response of the cells to MTX in folate added medium, but it had a major impact on the cellular response in folate depleted medium. The association between UNG genotype and folate status in the response to MTX, might be attributed to the mechanism by which MTX induces DNA damage. MTX inhibits the activity of DHFR promoting the accumulation of DHF, which is capable of reactivating DHFR. However, in the absence of folate DHF concentration is reduced, where DHF will not be competing with MTX to reactivate DHFR.

In addition, we observed the impact of Ung genotype on the cell cycle progression. The variation in the doubling time of $UNG^{-/-}$ and $UNG^{+/+}$ MEFs was insignificant, which suggests that UNG deletion doesn't impact cell cycle progression in the presence of folate

in the culture media. However, we observed a pronounced impact of Ung genotype on cell cycle progression in cells cultured in a folate deficient medium, where the doubling time of $Ung^{-/-}$ was greater than that of $Ung^{+/+}$ MEFs. We can infer that Ung genotype impacts cell cycle progression when folate is depleted, which suggests a possible synergistic effect of folate depletion and Ung deletion.

It has been suggested by Bulgar et al that UNG increases the cells resistance to some cytotoxic agents (12). They demonstrated that 5-flouro Uracil, pemetrexed, TMZ, and cisplatin had a more cytotoxic effect on $UNG^{-/-}$ DLD1 colon cells (in contrast to the $UNG^{+/+}$ cells). The increased sensitivity of $UNG^{-/-}$ cells was related to the lack of the UDG enzyme activity. This conclusion opposes what we proposed regarding the role of UNG in the cells response to MTX and H_2O_2.

A possible explanation for our observation regarding the impact of UNG on the cells sensitivity to MTX and H_2O_2, is that the removal of the misincorporated Uracil by the UDG in the presence of cytotoxic drugs promotes the cytotoxic effect of the drugs, and thus the removal of uracil seems to be more harmful to the cells than its accumulation. Looking at the doubling time data, we can deduce that UNG deletion stalls cell cycle progression, which might be associated to the decreased mutagenic impact of UNG deletion. Moreover, the removal of the Uracil results in the formation of lesions in the DNA known as abasic sites, which are more detrimental to the cell than the misincorporated Uracil; these lesions are known to be mutagenic and toxic, and to delay the cell cycle progression (47).

MTX treatment leads to uracil accumulation, and induces the activity of UDG beyond the normal physiological levels to remove the excess misincorporated uracil, promoting the

formation of abasic sites. Elder et al examined the role of overexpression of fission yeast homologue of the human UDG in causing DNA damage. In this study Ung 1 (from the fission yeast) and UNG2 (the nuclear isoform of the human UNG gene) were found to be closely related (32). According to this study, ung1 overexpression lead to a significant increase in the number of abasic sites formed which leads to a delay in the cell cycle progression and leads to cell death; this delay is associated with the activation of the checkpoints at different stages of the cell cycle due to the accumulation of the DNA damage. Even the lowest level of ung1 induction lead to a significant increase in the number of abasic sites formed (32).

On the other hand, our doubling time data suggests that the deletion of UNG resulting in uracil accumulation, delays cell cycle progression. We can infer that uracil accumulation slows down the growth of the cell, but it is less harmful to the cell than the formation of abasic sites in the presence of MTX and H_2O_2.

Moreover, it was suggested that the deletion of UNG improves the viability of cells in Saccharomyces cerevisiae that cannot survive the detrimental outcome of the abasic sites; these cells lack APN1, APN2, and RAD1 which are responsible for the AP endonuclease activity in Saccharomyces cerevisiae (49). The decline in the lethality observed as a result of UNG deletion was compared to that of deleting Ogg1, Mag1, Ntg1, and Ntg2, which are enzymes involved in the BER repair pathway. Lethality of the yeast cells declined to a greater extent as a result of UNG deletion; therefore validating our observation regarding the detrimental effect of Uracil removal by UDG and the formation of abasic site (49).

When we treated $UNG^{+/+}$ cells with MTX, we detected an increase in uracil in DNA compared to that of untreated $UNG^{+/+}$ cells, which demonstrates that MTX promotes the

accumulation of uracil in the DNA and thus provides us with the possible explanation behind the decreased cell viability in the presence of MTX. By comparing the uracil accumulation in DNA of MTX treated $UNG^{+/+}$ and $UNG^{-/-}$, we noticed an increase in uracil accumulation in $UNG^{-/-}$ compared to the MTX treated $UNG^{+/+}$ cells; however, the % of viable cells was higher in MTX treated $UNG^{-/-}$ cells. This further confirms our previous deduction regarding the role of UDG in the enhancement of the damaging impact of MTX, and that the increased accumulation of uracil as result of MTX treatment is less damaging to the DNA than the removal of the uracil, which promotes uracil accumulation.

The role of folate in preserving the cellular genomic integrity has been examined in depth. In our study we detected the impact of folate deficiency on cell growth by comparing at the doubling times of FA and FD cells, and we established that folate deficiency stalls the growth of cells. The mechanism through which folate deficiency delays the cell cycle progression is associated to the increase in uracil; this has been demonstrated in our data, where uracil accumulation in the folate depleted MEFs was significantly greater than that of folate added MEFs. . It has been established that folate deficiency induces DNA damage due to a defect in the DNA repair pathway. Folate deficiency has been associated with a decreased induction of the BER pathway; this decrease in the induction in the BER pathway induces the accumulation of uracil which has been associated with a delay in cellular proliferation (40).

The role of folate deficiency in colorectal cancer has also been studied, and it was established that folate intake is associated with risk of colorectal cancer; sufficient folate intake was correlated to a decrease in the risk of colorectal cancer development (43-44). According to our data, folate deficiency causes an increase in uracil accumulation, which

can be related to the decreased ability of the cell to convert dUTP to dTTP inducing DNA damage, and as explained above reduces the ability to repair the damage, which has been associated with increased risk of colorectal cancer (42). To further demonstrate the impact of folate deficiency on the BER pathway, it has been showed that folate deficiency reduces colon cancer development in β-pol deficient mice by reducing the excision repair capacity and accumulating the damage; thus promoting cell death instead of cell survival (34).

In our study, we detected a decreased ability to repair the DNA damage induced by the introduction of H_2O_2 and MTX; therefore folate deficiency is capable of producing damage to the cell, comparable to that of MTX and H_2O_2. Furthermore, it has been shown that folate deficiency acts synergistically with other DNA damaging agents, which explains the reason behind the enhanced cytotoxic effect of MTX and H_2O_2.

Chapter 6

Conclusion

In our study we established that folate and uracil-DNA glycosylase affect the ability of the cells to resist DNA damage, which results in the delay of cell cycle progression and the induction of uracil accumulation in the cell. Furthermore, we observed the detrimental effect of MTX and H_2O_2 on the viability of the cells, and the impact of Ung genotype and folate depletion on the cellular response to these drugs.

Our preliminary data showed that MTX induces uracil accumulation; however due to experimental errors we failed to see a significant difference in uracil accumulation in the control and MTX treated group.

Therefore, further research is required in order to determine the mechanism through which MTX and H_2O_2 induce DNA damage and cell apoptosis**,** and to explain the reason behind the increased resistance of the cells as a result of Ung deletion. We also need to determine the impact of MTX and H_2O_2 on UDG activity and other enzymes involved in the BER pathway to show the impact of those two drugs on the ability of the cell to repair itself.

APENDIX A

Table 1. 1 Human DNA glycosylases for oxidative base damage (50)

DNA glycosylase	Substrates	Type
MPG	3-meA, 7-meA, 3-meG, 8-oxoG	monofunctional
UNG	U, 5-fU	monofunctional
hOGG1	8-oxoG, FapyG	bifunctional
hNTH1	Tg, hoU, hoC, urea, FapyG	bifunctional
hNEIL1	Tg, hoU, hoC, urea, FapyG, FapyA	bifunctional
hNEIL2	AP site, hoU	
hMYH	A, 8-oxoG	monofunctional
hSMUG1	U, hoU, hmU, fU	monofunctional
TDG	U, C, G	monofunctional
MBD4	5meC, T, fU	monofunctional

Figure 1. 1 Alkylated and halogenated bases (5)

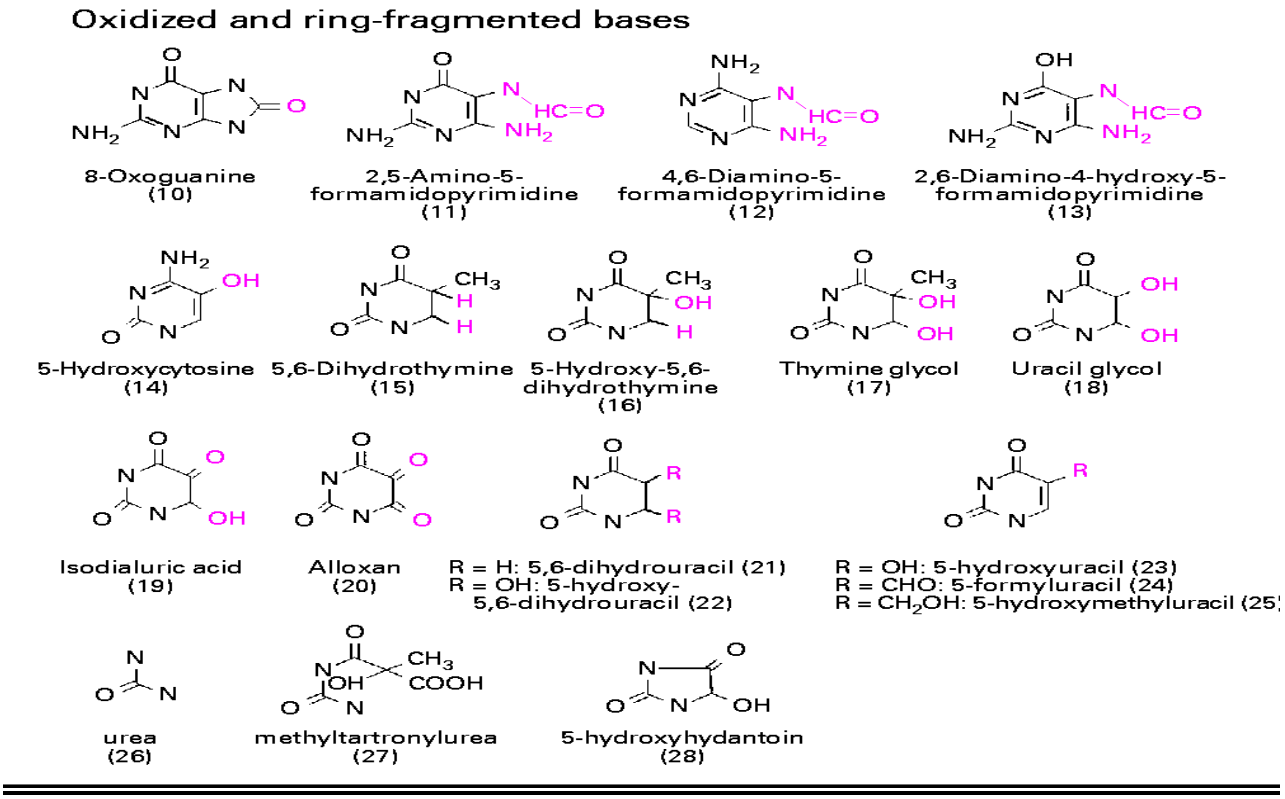


Figure 1. 2 **The process of H_2O_2 detoxification (Cabelof et al)**

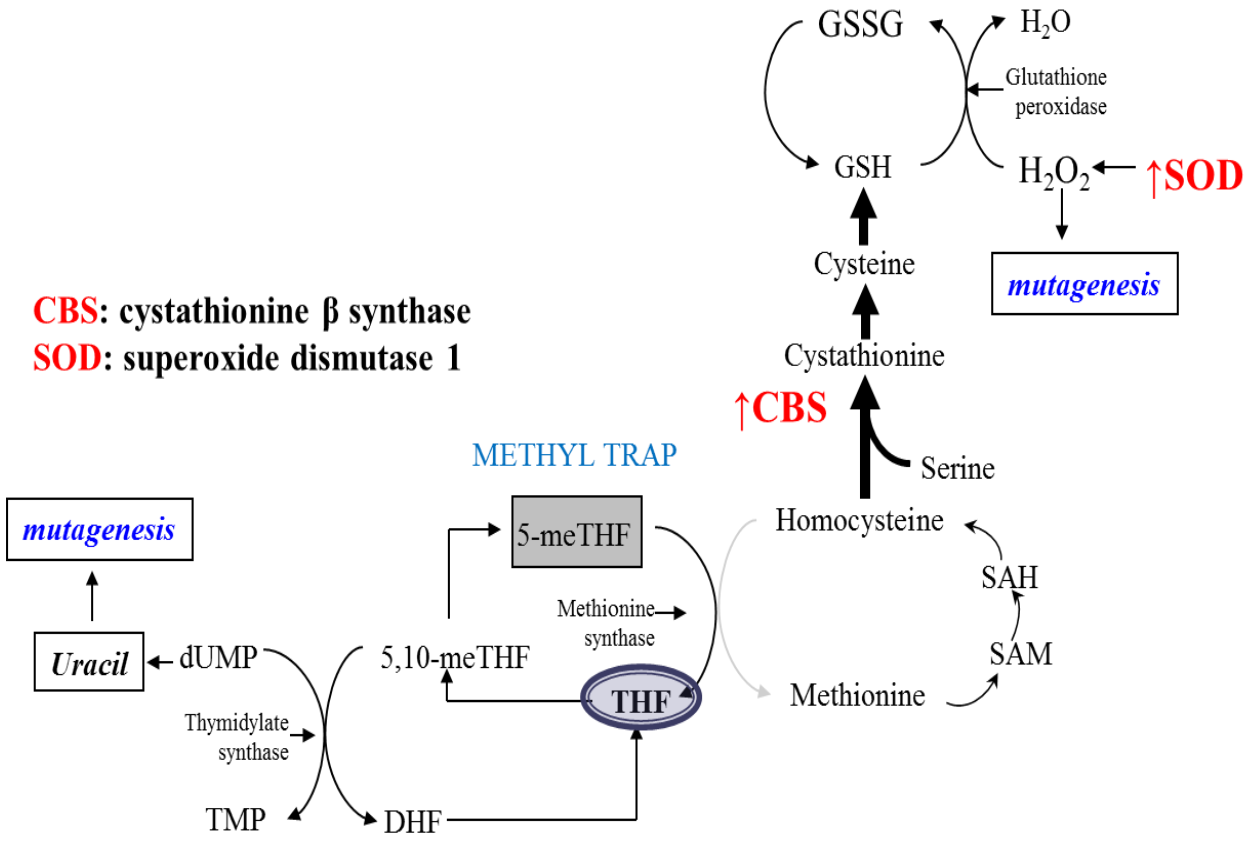


Figure 1. 3 Mechanism of Action of Methotrexate (15)

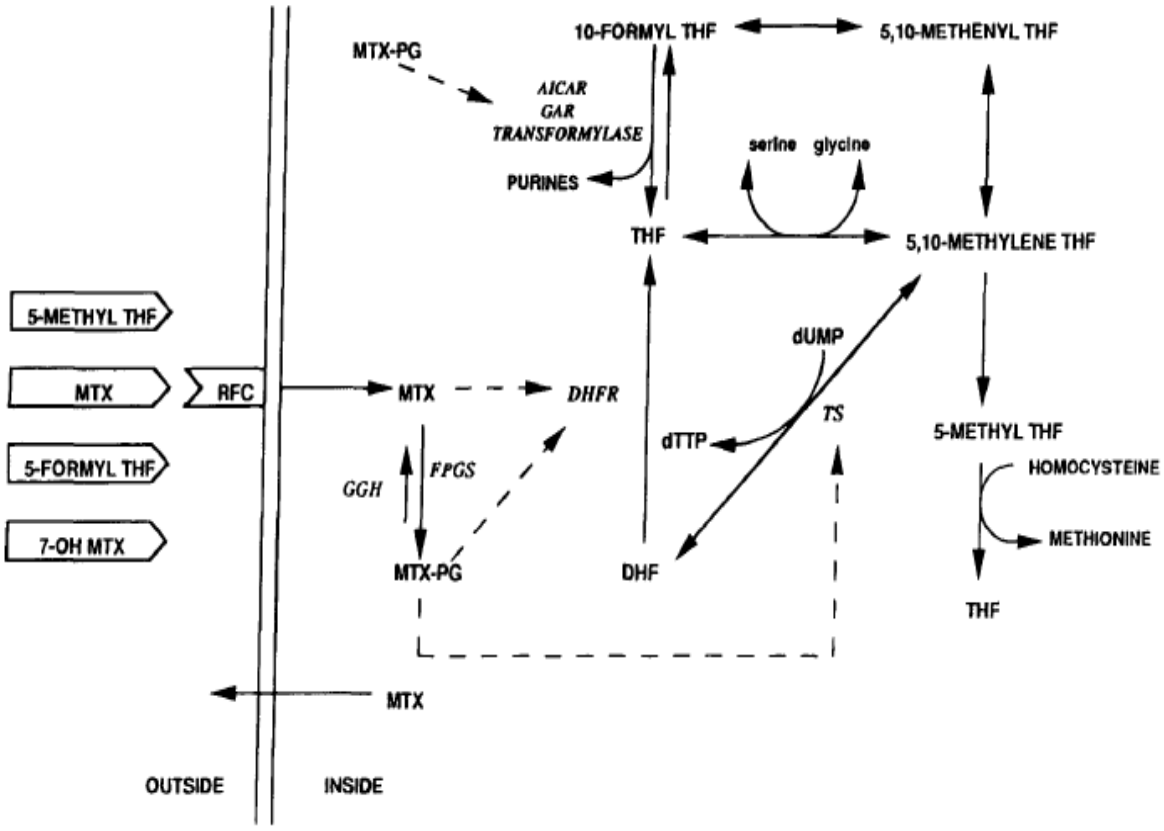


Figure 1. 4 Base excision repair pathway

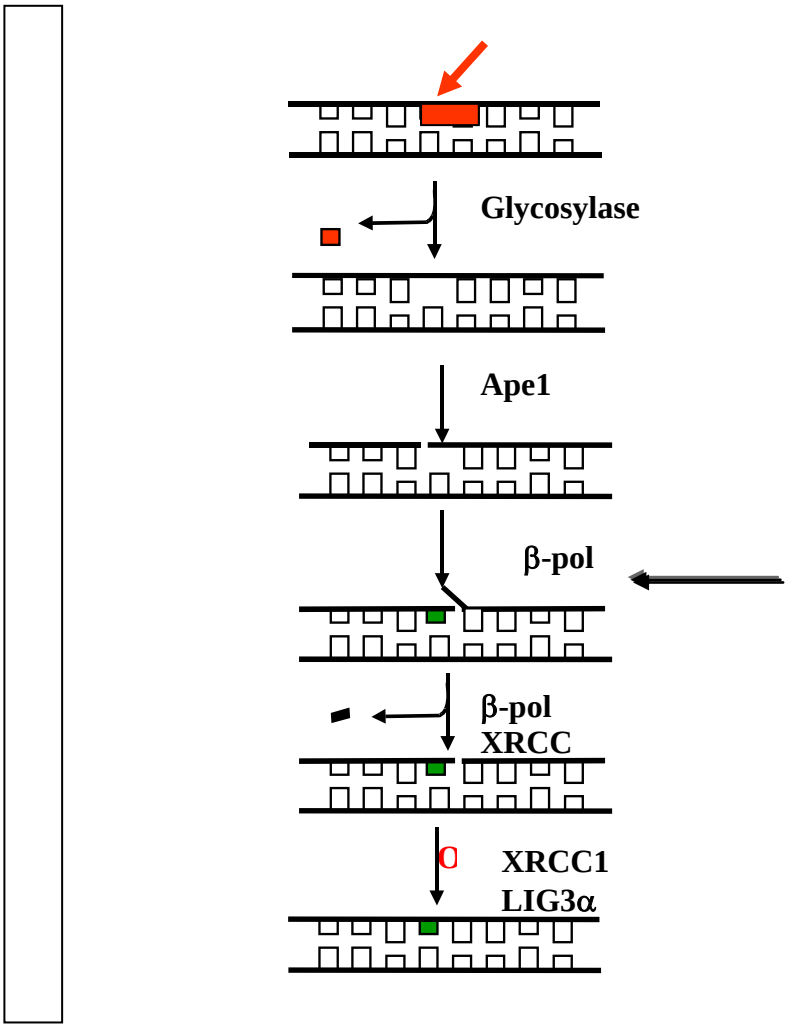
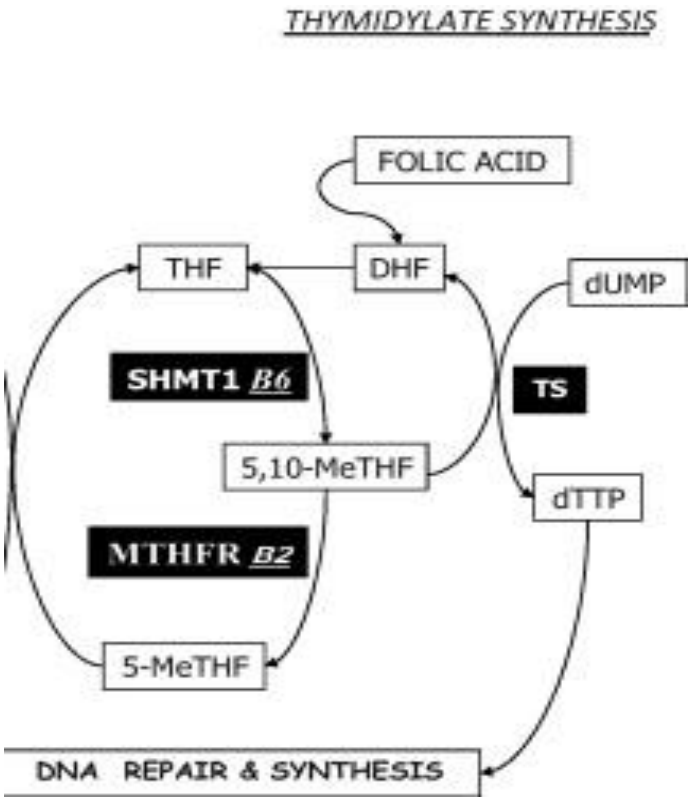


Figure 1. 5 Thymidylate synthesis



REFERENCES

(1) Unnikrishnan A, Raffoul JJ, Patel HV, Prychitko TM, Anyangwe N, Meira LB, Friedberg EC, Cabelof DC, Heydari AR. Oxidative stress alters base excision repair pathway and increases apoptotic response in apurinic /apyrimidinic endonuclease 1/ redox factor-1 haploinsufficient. Free Radical Biology and Medicine 2009. 46(11):1488-99

(2) Cabelof et al. Attenuation of DNA polymerase B-dependent base excision repair and increased DMS induced mutagenicity in aged mice. Mutat Res. 2002;500(1-2):135-45

(3) Cabelof et al. Caloric restriction promotes genomic stability by induction of base excision repair and reversal of its age-related decline. DNA Repair (Amst). 2003;2(3):295-307.

(4) Basso D, Navaglia F, Fogar P, Zambon CF, Greco E, Schiavon S, Fasolo M, Stranges A, Falda A, Padoan A, Fadi E, Pedrazzoli S, Plebani M. DNA repair pathways and mitochondrial DNA mutations in gastrointestinal carcinogenesis. ClinChimActa. 2007; 381(1):50-5.

(5) Slupphaug G, Kavli B, Krokan HE. The interacting pathways for prevention and repair of oxidative DNA damage.Mutat Res. 2003;531(1-2):231-51

(6) Cabelof et al. Imbalanced base excision repair in response to folate deficiency is accelerated by B-pol Haplosufficiency (Diane) CANCER RESEARCH. 2003;(63):5799-5807

(7) Stanczyk et al. Role of base-excision repair in the treatment of childhood acute lymphoblastic leukaemia with 6-mercaptopurine and high doses of methotrexate. Muta Res. 2012;(741):13-21

(8) Welsh et al. Expression of uracil DNA glycosylase (UDG) does not affect cellular sensitivity to thymidylate synthase (TS) inhibition.Eeuropean Journal of Cance. 2003;(39):378-387

(9) French et al. Folate (vitamin B9) and vitamin B12 and their function in the of nuclear and mitochondrial genome integrity. Muta Res. 2011

(10) Stanger O, Wonisch W. Enzymatic and Non-enzymatic Antioxidative Effects of Folic Acid and Its Reduced Derivates. Subcellular Biochemistry ;(56)

(11) Cabelof et al. A Sensitive Biochemical Assay for the Detection of Uracil (Diane). Enviromental and Molecular Mutagenesis. 2006;(47):31-37

(12) Bulgar et al. Removal of uracil by uracil DNA glycosylase limits pemetrexed cytotoxicity: overriding the limit with methoxyamine to inhibit base excision repair. Cell Death and Disease (2012) 3, e252

(13) Frosina et al. Overexpression of enzymes that repair endogenous damage to DNA. Eur. J. Biochem. 2000;(267):2135-2149

(14) Goulian et al. Methotrexate-induced misincorporation of uracil into DNA. Biochem. 1980;(Vol.77 No.4):1956-1960

(15) Matherly LH, Taub JW. Methotrexate Pharmacology and Resistance in Childhood Acute Lymphoblastic leukemia. Leukemia and Lymphoma. 1996;(Vol. 21):359-368

(16) Goulian M, Bleil B, Tsang BY,. The effect of Methotrexate on levels of dUTP in Animal Cells. The Journal of Biologocal Chemsitry. 1980;(Vol. 255, No 22):10630-10637

(17) Ward J, Evan JW, Limoli C.L, Calabro-Jones PM, Radiation and Hydrogen peroxide induced free radical damage to DNA. Br J Cancer Suppl. 1987 Jun;8:105-12.

(18) Spector A, Kleiman NJ, Huang RRC, Wang RR,. Repair of H_2O_2- Inducced Damage in Bovine Lens Epithelial Cell Cultures. Exp.Eye Res.1989;(49):685-698

(19) Krohn K, Maier J, Paschke R,. Mechanisms of Disease: Hydrogen Peroxide, DNA Damage and Mutagenesis in the Development of Thyroid Tumor. Nature Clinical Practice Endocrinology & Metabolism. 2007 Vol.3 No.10

(20) Jaruga P, Dizdaroglu M,. Repair of products of oxidative DNA base damage in human cells. Nucleic acids Research. 1996 Vol.24, No. 8, 1389-1394

(21) Cabelof et al. Ageing and base excision repair: In need of a comprehensive approach. DNA Repair . 2007;(6) 1399-1402

(22) Xavier AC, Edwards H, Dombkowski A, Balci TB, Berman LN, Dellaire G, xie C, Buck SA, Matherly LH, Ge Y, and Taub JW,. A Unique Role of GATA1 in Down Syndrome Acute Megakaryocytic Leukemia Biology and Therapy. PLoS One. 2011;(Vol.6 Issue):e27486

(23) Courtemanch C, Huang AC, Schwab HI, Kerry N, Ng BY, and Ames BN,. Folate Deficiency and Ionizing radiation cause DNA breaks in primary human lymphocytes: a comparison. The FASEB Journal express article. 2003; 10.1096/fj-03-0382fje.

(24) Simon et al. Transcriptional profiling of the age-related response to genotoxic stress points to differential DNA damage response with age. Mechanisms of Ageing and Development. 2009;(130) 637-647

(25) Cabelof et al. Age-Related Loss of the DNA Repair Response Following Exposure to Oxidative stress. Journal of Gerontology: BIOLOGICAL SCIENCES. 2006;Vol.61A No.5, 427-434

(26) Cabelof DC, Ikeno Y, Nyska A, Busuttil RA, Anyangwe N, Vijg J, Matherly LH, Tuckerr JD, Wilson SH, Richardson A, and Heydari AR,. Haplosufficiency in DNA Polymerase β Increases Cancer Risk with Age and Alters Mortality Rate. Cancer Res. 2006;66:(15)

(27) Cabelof DC, Guo ZM, Raffoul JJ, Sobol RW, Wilson SH, Richardson A, and Heydari AR,. Base Excision Repair Deficiency Caused by polymerase β Haploinsufficiency: Accelerated DNA Damage and Increased Mutational Response to Carcinogen. Cancer Res. 2003;(63):5799-5807

(28) Kohli M, and Jorgensen TJ,. The Influence of SV40 Immortalization of Human Fibroblasts on p53-Dependent Radiation Responses. BIOCHEMICAL AND BIOPHSYSICAL RESEARCH COMMUNICATION. 1999; Vol. 257 No. 1.

(29) Genstier et al. Mechanisms of Action of methotrexate. Immunopharmacology. 2000;(47):247-257

(30) Segal R, Yaron M, and Tartakovsky B,. Methotrexate: Mechanism of Action in Rheumatoid Arthritis. Seminar in Arthritis and Rheumatism. 1990; Vol. 20 No.3: 190-199

(31) Colleoni et al. Dose-Response Effect of Adjuvant Cyclophosphamide, Methotrexate, 5-Fluorouracil (CMF) in Node-positive Breast cancer. European Journal of Cancer. 1998 Vol. 34, No.11 1693-1700

(32) Elder RT, Zhu X, Priet S, Chen M, Yu M, Navarro JM, Sire J, Zhao Y. A fission yeast homologue of the human uracil-DNA-glycosylase and their roles in causing DNA damage after overexpression. Biochem Biophys Res Commun. 2003 Jul 4;306(3):693-700.

(33) Ventrella-Lucente LF, Unnikrishnan A, Pilling AB, Patel HV, Kushwaha D, Dombkowski AA, Schmelz EM, Cabelof DC, and Heydari AR,. Folate Deficiency Provides Protection Against Colon Carcinogenesis in DNA Polymerase β Haploinsufficient Mice. The JOURNAL OF BIOCHEMISTRY. 2007;(18):541-552

(34) Katula et al. Folate deficiency in normal human fibroblasts leads to altered expression of genes primarily linked to cell signaling, the cytoskeleton, and extracellular matrix. Journal of Nutritional Biochemistry. 2007;(18) 541-552

(35) Krokan HE, Standal R, and Slupphaug G,. DNA glycosylases in the base excision repair of DNA. Biochem.J. 1997;(325)1-16

(36) Kronenberg et al. Folate Deficiency Induces Neurodegeneration and Brain Dysfunction in Mice Lacking Uracil DNA Glycosylase. The Journal of Neuroscience. 2008; 28(28):7219-7230

(37) Ide H, and Kotera M. Human DNA glycosylases involved in the Repair of Oxidatively Damaged DNA. Biol.Pharm. Bull. 2004; 27(4):480-485

(38) Unnikrishnan et al. Folate deficiency regulates expression of DNA polymerase β in response to oxidative stress. Free Redical Biology & Medicine 2011;(50): 270-280

(39) Duthie, S.J., et al., Folate deficiency in vitro induces uracil misincorporation and DNA hypomethylation and inhibits DNA excision repair in immortalized normal human colon epithelial cells. Nutr Cancer, 2000. **37**(2): p. 245-51.

(40) Duthie, S.J. and A. Hawdon, DNA instability (strand breakage, uracil misincorporation, and defective repair) is increased by folic acid depletion in human lymphocytes in vitro. FASEB J, 1998. **12**(14): p. 1491-7.

(41) Singh R, Fouladi-Nashta AA, Li D, Halliday N, Barrett DA, Sinclair KD. Methotrexate Induced Differentiation in Colon Cancer Cells is Primarily Due to Purine Deprivation. J Cell Biochem. 2006 Sep 1; 99(1):146-55.

(42) Guillet M, Boiteux S. Origin of Endogenous DNA Abasic Sites in Saccharomyces cerevisiae. Mol Cell Biol. 2003 Nov;23(22):8386-94.

(43) Cravo, M. L., Mason, J. B., Dayal, Y., Hutchinson, M., Smith, D., Selhub, J., and Rosenberg, I. H. Folate deficiency enhances the development of colonic neoplasia in dimethylhydrazine-treated rats. (1992) Cancer Res. 52, 5002–5006

(44) Kim, Y. I., Salomon, R. N., Graeme-Cook, F., Choi, S. W., Smith, D. E., Dallal, G. E., and Mason, J. B. Dietary folate protects against the development of macroscopic colonic neoplasia in a dose responsive manner in rats. (1996) Gut 39, 732–740

(45) Hoover, K. L., Lynch, P. H., and Poirier, L. A. Profound postinitiation enhancement by short-term severe methionine, choline, vitamin B12, and folate deficiency of hepatocarcinogenesis in F344 rats given a single low-dose diethylnitrosamine injection. (1984) J. Natl. Cancer Inst.73, 1327–1336

(46) James, S. J., Miller, B. J., Basnakian, A. G., Pogribny, I. P., Pogribna, M., and Muskhelishvili, L. Apoptosis and proliferation under conditions of deoxynucleotide

pool imbalance in liver of folate/methyl deficient rats. (1997) Carcinogenesis 18, 287–293.

(47) Guillet M, Boiteux S. Origin of Endogenous DNA Abasic Sites in Saccharomyces cerevisiae. Mol Cell Biol. 2003 Nov; 23(22):8386-94.

(48) Blount, B. C., Mack, M. M., Wehr, C. M., MacGregor, J. T., Hiatt, R. A., Wang, G., Wickramasinghe, S. N., Everson, R. B., and Ames, B. N. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage (1997). Proc. Natl. Acad. Sci. USA **94,** 3290-3295

(49) Kronenberg, G., et al., Folate deficiency induces neurodegeneration and brain dysfunction in mice lacking uracil DNA glycosylase. J Neurosci, 2008. **28**(28): p. 7219-30.

(50) Ide H, Kotera M. "Human DNA glycosylases involved in the repair of oxidatively damaged DNA". Biol. Pharm. Bull (2004). **27** (4): 480–5.

ABSTRACT

ROLE OF URACIL DNA-GLYCOSYLASE AND FOLATE IN THE REPAIR OF OXIDATIVE DAMAGE

by

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The impact of DNA damage on genomic integrity has been widely investigated due to its association with cancer and ageing. DNA repair pathways play a critical role in preserving the genomic integrity and protecting the cells against DNA damage caused by oxidative stress and folate deficiency. Base Excision Repair (BER) is one of the major pathways involved in the cellular response to DNA damage, primarily the damage caused by oxidative stress. UNG genotype and folate were found to have an impact on the ability of the cell to repair DNA damage.

The present study was designed to examine the impact of uracil-DNA-glycosylase and folate deficiency on the cell cycle progression and the cells capacity to repair DNA damage in the presence of carcinogenic agents such as H_2O_2 and MTX, using the doubling time, cytotoxicity, and uracil accumulation assays. Based on our data, we can conclude that folate deficiency and UNG genotype stalls cell cycle progression, induces uracil accumulation, and reduces the cells ability to repair the DNA damage.

AUTOBIOGRAPHICAL STATEMENT

Sarah T. Dubaisi graduated in 2010 from the American University of Beirut with a Bachelor of Science Degree in Nutrition and Food Sciences. In the fall 2010, she entered the Master of Science program in the department of Nutrition and Food Science in Wayne State University.

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Sarah participated in poster sessions in the Midwest DNA repair Symposium (2011 and 2012) which were held in the University of Toledo and University of Cincinnati, OH.